

XLI.—*A Contribution to the Chemistry and Physiology of Foliage Leaves.*

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PART I.—THE STARCH AND DIASTASE OF LEAVES.

1. *Introduction.*

THE investigation which we have recorded in this paper is an attempt to throw some light on the occurrence, relations, and physiological significance of the starch, diastase, and sugars of foliage leaves.

It originated in an attempt to explain a certain technical operation in brewing which had been hitherto purely empirical in its applica

tion, and to which it is perhaps desirable to refer briefly, owing to a certain amount of chemical interest attached to it.

When the primary fermentation process is completed, and the beer is racked into casks, it has been customary in some localities to add to the finished beer a small quantity of dry hops, the effect of which is very marked in several ways. As to the direct influence of these hops on the clarification of the beer, nothing need be said here, nor is it necessary to refer to their antiseptic action. There is, however, another strongly marked effect which has been generally recognised for generations, but has never, up to the present time, received any adequate explanation. We refer to the secondary fermentation, which is undoubtedly hastened by the addition of these hops. It is altogether unnecessary for us to give any details of the investigation which led us to a true explanation of this effect, as we have recently treated of it at length elsewhere (*Trans. Institute of Brewing*, 1893, 6, 94—104), but we may briefly state that the results of the technical process alluded to were found to depend on the presence in the hop-strobiles of a small but very appreciable amount of a *diastase*, sufficient to slowly hydrolyse the non-crystallisable products of starch-transformation left in the beer, and to reduce them to a condition in which they can be seized upon and fermented by the yeast.

The interesting question now arose whether this occurrence of diastase in the hop-strobile is an isolated case, or is a special example of a widely distributed property of vegetable tissue.

An examination of all the published facts relating to the occurrence of diastase in plants revealed a curious number of conflicting statements from which it was impossible to draw any conclusion without further investigation. This investigation it was considered desirable to make, as the subject is closely connected with some of our previous work, and is of the first importance for a proper understanding of many problems presented by vegetable physiology.

As the work progressed we found ourselves carried by degrees into all the vexed questions connected with the first formation of starch in the foliage-leaf, the mode of its dissolution and translocation in the plant, and the nature of the intermediate products. In such an investigation it is impossible to draw a line beyond which one will not advance, as the subject is a never-ending one, but as we found ourselves being urged along lines of research somewhat divergent from those we have marked out for ourselves during the last few years, we considered it better no longer to delay the publication of our results, which, though in many respects incomplete, we think will not prove to be devoid of interest both to the chemist and vegetable physiologist.

In the following historical *résumé* we give, in as short a space as

possible, an account of the more important work of previous observers, in so far as it is necessary for the understanding of that which follows. The student who desires to fully acquaint himself with the voluminous literature of the subject is referred to the complete list of original memoirs given in the bibliography at the end of this paper.

References in the text to the original papers are made through the serial numbers of this bibliographical list.

2. *Historical.*

It is to the year 1837 that we have to refer the first recorded observation of the occurrence of starch-granules as inclusions in the chlorophyll-bodies of green plants. In that year, von Mohl (1) published his epoch-making dissertation on the structure of the chlorophyll-granules, in which for the first time he distinguished the inclusions in those bodies from the actual substance of the chlorophyll-granule itself. Small *starch-granules* were frequently, but not invariably, observed within the chlorophyll-body, and these were found to be capable of being differentiated from the ground-mass in which they were embedded by means of iodine.

Sixteen years afterwards, in 1853-54, these observations were confirmed by C. Cramer, whose results are quoted by C. Nägeli, at p. 399 of his great work, *Die Stärkekörner* (5), but we have been unable to obtain the full reference to these papers.

Nägeli himself also gives in the same monograph his own observations in this direction, and has appended drawings of starch-grains included in the chloroplasts of leaves of *Begonia* and *Nephrolepis exaltata*.

In 1866, the subject was independently investigated by J. A. Böhm (4), and in the following year by A. Gris (3). Both these observers recognise the correctness of previous observations, and Böhm employs for the first time the now well-known method of detecting minute starch-granules by successive treatment of the tissue with caustic potash and iodine. He also noticed that there are certain plants, *e.g.*, *Asphodelus luteus*, *Allium fistulosum*, and *Orchis militaris*, whose chloroplasts are incapable of forming starch within their substance.

So far all the more recent observations had distinctly shown that the starch-granule is a product of the chloroplast, and that the view which had been promulgated by Mulder, that the chloroplast is the child of the starch-granule, is untenable. Although, however, it was recognised that the occurrence of starch in the chloroplasts was a secondary phenomenon, it does not appear to have been suspected

that there was any very distinct relation between this starch and the processes of assimilation. It was left to Sachs to take this great step in advance. In 1862, he published (6) his important memoir dealing with the influence of light on the production of starch in the chlorophyll-granule, and two years later this was followed by another paper (7) on the subject. Sachs here clearly establishes the important fact that the appearance of starch in the chlorophyll-granule is induced by, and is dependent on, the action of light of sufficient intensity, and that the green colouring-matter of the chloroplast is as essential to the production of this autochthonous starch as it is for the decomposition of carbon dioxide, for the decolourised chloroplasts of an etiolated plant have not the power, as long as they remain colourless, to produce starch within their substance. Sachs in fact, for the first time clearly formulates the proposition that the production of starch in the chlorophyll-granule is directly connected with assimilation. He, moreover, shows that when plants are put in the dark the starch disappears from their chloroplasts, to reappear again when the plants are once more illuminated. These facts, he rightly considers, are fraught with the weightiest consequences for the theory of assimilation, as they indicate a daily periodic change in green leaves, the starch which is formed in the chloroplasts during the hours of daylight being wholly or partially redissolved and removed from the leaf during the night, to supply the constant demands of the growing parts of the plant.

These ideas, as we shall see later on, were elaborated and expanded still further in a later paper by Sachs.

In 1873, further important contributions were made to the subject by Godlewski (11) and by Pfeffer (10), who proved in experiments on *Raphanus sativus* and *cress*, that when the plants were placed in air entirely deprived of carbon dioxide, *i.e.*, in an atmosphere in which no assimilation could take place, no starch was formed in the chloroplasts, even when the plants were exposed to intense light. Godlewski moreover found that previously-formed starch completely disappeared from the chloroplasts under these conditions, and, on the other hand, that starch-formation within these bodies could be materially accelerated by increasing, within certain limits, the amount of carbon dioxide in the air around the plant.

These results strengthened to a remarkable extent the earlier ideas of Sachs, that the formation of autochthonous starch and assimilation go hand in hand.

A little later, J. A. Böhm (12), (13), (14) gave a very necessary caution against considering *all* starch which occurs in the chloroplasts of green tissue as the immediate product of assimilation, *i.e.*, autochthonous. He found, when experimenting with seedlings of

Raphanus sativus, *Lepidium sativum*, and *Phaseolus multiflorus*, still containing a certain amount of the reserve material of the seed within their tissues, that a decided transfer of some of these materials could be traced to the chloroplasts of the leaf, where there was a distinct production of starch, and that this took place both under conditions of feeble light-intensity and also in the entire absence of carbon dioxide, thus disposing of the possibility of the starch production being due to assimilation. He concludes that the formation of starch in chlorophyll-granules is, in many cases, the result of a metamorphosis of bodies not intrinsic to the cells in which the accumulation takes place, and that in such cases the starch owes its origin to materials elaborated by the plant elsewhere.

In 1880 a flood of light was thrown upon this subject by Schimper (20), who, in this year, published the results of his classical work upon the development of the starch-granule in plants.

Commencing with a very complete study of the mode of formation of starch in the special organs of assimilation, the chlorophyll-bodies, he confirmed, in many respects, the observations of the earlier workers, and carried our knowledge a step further by indicating how closely the shape of the starch-granule is dependent upon the shape of the chlorophyll-body which gives rise to it, and upon the position it occupies with regard to the chlorophyll-body during its development. But it is in his study of the growth of the starch-grains within those parts of the plant which contain little or no chlorophyll that Schimper makes his most original and important observations. He finds that in all parts of the plant in which starch is being deposited, either as reserve- or transitory-starch, the starch-granules in process of development are not surrounded immediately by ordinary protoplasm, but are contained in, or attached to, peculiar refrangible corpuscles, which are spherical or spindle-shaped. These are the "*Stärkebildner*," the starch-forming corpuscles or amyloplasts, which are causally related to the deposition of the starch-granules in the non-chlorophyllous parts of the plant, just as are the chlorophyll bodies, on the other hand, in the green assimilating cells. The amyloplasts are, in fact, the starch-forming organs of the cells which do not assimilate.

The point which is of greatest importance to us in this work of Schimper's at the present moment is the close analogy which he points out as existing between the amyloplasts and the chloroplasts as regards structure, development, and starch-producing function. It is, in fact, more than a mere analogy, for Schimper observed that amyloplasts may, under favourable conditions of light, be actually converted into chlorophyll-corpuscles capable of assimilating in the usual manner, and that, in fact, this conversion takes place normally

and regularly in the development of a plant organ. Schimper also attempted to determine if true chlorophyll-corpuscles are capable of forming starch out of ready-formed and assimilated materials. This is perhaps the least satisfactory portion of this important memoir. He approached the question by instituting the following experiment. A root-stock of *Tradescantia rubella* was kept in the dark until the large starch-grains had disappeared from the chlorophyll-corpuscles of the mesophyll; it was then exposed to light of sufficient intensity to effect the re-formation of normal chlorophyll-corpuscles without producing starch within them by the assimilative process. It was found that the chlorophyll-corpuscles of the bundle-sheaths of the leaves and of the parenchyma of the stem had been able to form starch at the expense of the general reserve-materials of the root-stock, but that the chloroplasts of the mesophyll had not been able to elaborate any starch from the same store. From this Schimper concludes that although some chloroplasts, like those of the parenchyma of the stem and of the vascular bundles of the leaves, have the power of producing starch from ready-formed substances, such is not the case with the chloroplasts of the spongy and palisade parenchyma of the mesophyll, which is only able to produce its starch by assimilation from the atmosphere.*

This one supposed difference of function between the chloroplasts of the leaf-parenchyma and the ordinary colourless amyloplasts was completely disposed of by the direct experiments of Böhm (30) in 1883, and a little later by those of A. Meyer (34 and 38). Both these observers found that leaf-parenchyma which has been fully depleted of starch will again form starch readily in the chloroplasts when the tissue is bathed with solutions of certain sugars. These observations have been frequently repeated by others, and we have, on many occasions, satisfied ourselves of their accuracy. We must therefore conclude that, as far as starch-formation is concerned, the chloroplasts of the mesophyll do not differ from the amyloplasts of the non-chlorophyllous parts of the plant as regards formation of starch from ready-formed sugars. The necessity for emphasising this important fact will be evident when, in a later part of this paper, we have to consider the mechanism of the dissolution and translocation of autochthonous starch.

In the year 1884, Sachs published his memorable paper "Ein Beitrag zur Kenntniss der Ernährungsthätigkeit der Blätter" (32), which must be regarded as a sequel to his papers of 1862 and 1864, to which reference has been already made. This paper considerably

* We must here not omit to mention the work of Dehnecke (19), "Ueber nicht assimilirende Chlorophyllkörner," which appeared as an Inaugural Dissertation in the same year in which Schimper's paper was published.

advanced our knowledge of the process of starch-formation in the chloroplasts of leaves, and threw much new light upon the conditions which govern the disappearance of this assimilated starch when it contributes to the normal metabolic processes of the plant. The importance of the paper is so great that we must consider it somewhat at length, especially as it has a very direct bearing upon our own work.

In order to form an estimate of the periodic variations in the amount of starch when the leaves are placed under various conditions, Sachs devised the following macroscopical method:—

The leaf was first immersed for a few minutes in boiling water, then completely decolourised with alcohol, and afterwards treated with a dilute iodine solution, the amount of starch present being judged by the intensity of the colouration produced in the leaf.

The varying amount of starch in the same leaf at different times of the day was estimated by applying this method to longitudinal strips cut from the leaf at intervals. The results so obtained not only confirmed in a remarkable manner the earlier observations of Sachs, that the starch formed in the leaf-chlorophyll under the influence of light disappears again in the dark or in deep shade, but also clearly showed that in plants grown under ordinary natural conditions the leaves during the night hours are rapidly depleted of the starch which they have accumulated during the day, the chlorophyll-bodies of the leaf tissue again elaborating starch with the return of daylight. The influence of sunlight and temperature on these periodic changes was also worked out, and it was shown that the rapidity of the disappearance of starch from the leaf during the night is a function of the temperature. For instance, with a night temperature of 9° C., leaves of *Helianthus*, *Solanum*, *Datura*, *Atropa*, and *Æsculus* were quite depleted of starch, whereas, under the same conditions, the leaves of *Phaseolus*, *Ampelopsis*, and *Aristolochia* were not fully depleted. As regards formation of starch during the day, this increases with the insolation and the temperature, so that, generally speaking, in clear weather during the summer the leaves which are poor in starch before mid-day are rich in it during the afternoon, and in the evening contain so much starch as to give a black metallic lustre with the iodine test. In order to form some idea of the actual weight of starch produced or lost by a leaf under these varying conditions, the variation in dry weight of a definite area of the leaf was determined. For this purpose it was necessary to employ plants having large leaves, such as *Helianthus annuus*, *Cucurbita Pepo*, or *Rheum officinale*. With the help of thin templates of board, rectangular pieces, having an area of 100 or 50 sq. cm., were cut from the half leaves under experiment. These pieces of leaf were first killed by submitting them to the action of steam for four or five minutes, in

order to avoid subsequent loss by respiration, and were then dried and weighed. From the dry weight of equal areas from the other halves of the leaves, submitted to the requisite conditions, the loss or gain in weight per square metre of leaf-surface was determined, and as this increase or deficiency was found to be correlative with the indications of the iodine test, the differences were attributed to the loss or gain of *starch* by the leaf. The following are some of the results which Sachs obtained by this method:—

Helianthus annuus.

	Grams of starch.
A. Loss per square metre of leaf in 10 hours of the night	9.64
Loss per hour per sq. m.....	0.964
B. Gain per square metre of leaf in 10 hours of the day	9.14
Gain per hour per sq. m.....	0.914

Cucurbita Pepo.

Gain per sq. m. in 3 hours of the day.....	2.03
“ “ per hour	0.68

Rheum officinale.

Gain per sq. m. in 5 hours of the day.....	3.26
“ “ per hour	0.652

When similar experiments were made by the insolation of plucked leaves, the petioles of which were immersed in water, the increase of weight was very much greater than when the leaf remained attached to the plant. This Sachs attributes to the assimilated starch accumulating in the leaf, owing to its products of dissolution not being able to flow out of the leaf into the stem. Under these conditions he found in *Helianthus* an increase of leaf-weight equal to 1.648 grams of starch per hour. As, however, we have reason to believe that under normal conditions depletion of the leaf through the petiole is going on simultaneously with the process of assimilation, the results tabulated above do not give in themselves a just idea of the total rate of assimilation. If we know the mean loss per hour during the 24 hours, the total assimilation would be approximately arrived at by adding this mean loss of weight, due to passage of materials out of the leaf, to the gain per hour during the day. Sachs has determined the loss per hour during the night, when no assimilation is going on, but justly remarks that there is good reason to believe the rate of loss by translocation during the day is somewhat greater than this, owing to the higher temperature; so that if we add the loss per hour during the night to the gain per hour during the day, we shall obtain

a result which will be somewhat, but not much, less than the true weight of the starch assimilated each hour of the day per square metre of leaf surface. In this way, Sachs found that the leaf of *Helianthus* assimilates about 1·8 grams, and that of *Cucurbita* about 1·5 grams, of starch per square metre per hour. This result agrees very well with those obtained by the insolation of plucked leaves, when the translocation of assimilated material is necessarily prevented.

By a further application of the method, and by measuring the total leaf-area of two experimental plants growing under very favourable conditions, Sachs found that, in a day of 15 hours, a *Helianthus* plant with 145 leaves of all sizes, and with a total leaf-area of 1·5 sq. m., assimilated 36 grams; whilst a large *Cucurbita* plant having a leaf-area of 7·3 sq. m. assimilated 185 grams in the same time.

To the portion of this important paper dealing with the mechanism of the dissolution of leaf-starch, and its translocation, we shall have occasion to refer more fully when we discuss the nature of the products of starch-dissolution, and their wandering within the plant.

Arthur Meyer, in 1885 (34), in an elaborate and suggestive paper on the assimilation-products of foliage-leaves, records the examination of a great number of plants for leaf-starch, using for the first time chloral hydrate and iodine as a microchemical test. As a general result, Meyer states that the dicotyledons store starch plentifully in their leaves, and the monocotyledons less so.

The relative ability of dicotyledons to produce leaf-starch is shown in a tabular form, the conclusions being based upon observations on from three to 20 species of each Natural Order.

Amongst monocotyledons, the *Gramineæ*, generally speaking, store much less starch in their leaves than do the *Dioscoreæ*, *Juncaceæ*, and *Alismaceæ*. The *Liliaceæ* are remarkable for storing very little starch, and in some cases none at all, e.g., *Allium Moly*, *A. Victorialis*, *A. spirale*, *A. sativum*, *A. odorum*, *A. Cepa*, *Scilla maritima*, &c.

An interesting investigation was then commenced to ascertain if this non-occurrence or scarcity of starch in the leaves of certain plants when assimilating is due to too rapid translocation of the assimilated products from the mesophyll as compared with the energy of assimilation. This question was answered by exposing cut leaves to bright light in an atmosphere containing 3 per cent. of carbon dioxide, i.e., under conditions most favourable to rapid assimilation and to the accumulation of the assimilated products within the leaf. As a rule, the plants whose leaves did not form starch under ordinary conditions did not do so when treated in the manner described, but two exceptions to this were found in *Hemero-*

callis flava and *Muscari moschatum*, whose leaves, ordinarily starch-free, produced starch in their chlorophyll-granules under these altered conditions. The general conclusions drawn are that the observed differences of production of starch in the leaves of plants are not due to the relatively rapid carrying away of the assimilated and reserve materials from the leaf.

In conjunction with this 1885 paper of Meyer, we must also consider an important one by the same author which appeared in the following year (38). It is here strongly insisted upon that, as regards starch-production, there is little or no difference between the assimilating cells of leaf-parenchyma and the parenchyma-cells of other organs. Any parenchyma-cells can assimilate when the *trophoplasts** have given rise to chlorophyll under the influence of light, and the whole paper affords the strongest possible evidence that the chloroplasts, the essential organs for assimilation of carbon from the air, are also capable of functioning like ordinary amyloplasts in elaborating starch from certain sugars and a few other substances.

Meyer also distinctly formulates for the first time the proposition that starch only arises in the leaf-cell if more of the nutritive starch-forming material is received than the leaf uses for its own nourishment and respiratory processes, and that the degree of concentration of the nutritive solution plays an important part in the result. Following in the main the experimental methods of Böhm, leaves which had been thoroughly depleted of their autochthonous and reserve-starch in darkness were divided into pieces of about 4—6 sq. cm. and floated on the nutritive solution, or, if the leaf was small, it was merely snipped round the margin to open the tracheïds, so that the solution might gain access to the parenchyma. It was found in this manner that *dextrose*, *levulose*, and *galactose* can be converted into starch by the leaf-parenchyma; but whilst there are some plants whose leaves can produce starch from all three kinds of sugar, this is not the case with all. Almost all leaves which are capable of forming starch at all produce it abundantly from a 10 per cent. solution of *levulose*, and a relatively small number from *dextrose*, whilst on the other hand only a very few leaves can form starch from *galactose*.

The general rule appears to be that those forms of sugar which are naturally present in the plant are favourable to starch-production in

* Meyer gives the collective and convenient name of *Trophoplasts* (= nurse-plastids) to the chlorophyll-granules generally, distinguishing the green-coloured assimilating organs, which are capable of making their starch directly from inorganic materials, as *autoplasts*, the colourless granules as *anoplasts* (= Stärkebildner or amyloplasts of Schimper), and as *chromoplasts* the granules which elaborate colouring matters other than chlorophyll. (*Vide Das Chlorophyllkorn*. A. Meyer, Leipzig, 1883.)

the leaf. A good instance of this is afforded by the *Compositæ*. This Natural Order contains *inulin* as a reserve-substance; the product of hydrolysis of inulin is levulose; consequently we find the leaves of the *Compositæ* especially well able to elaborate starch from levulose. On the other hand, from dextrose only small quantities of starch are elaborated by the leaf-cells of this order of plants, and none at all from galactose. Then, again, the *Sileneæ* naturally contain *galactose*, and we find in consequence abundant starch-formation in the leaves of these plants when they are floated in solutions of galactose.

The same holds good with regard to plants which contain *mannite*. Meyer also described some interesting observations on the value of cane-sugar, maltose, and glycerin as starch-forming nutrients.

We must also briefly refer to the work of E. Laurent (41) on the formation of starch in plants, for although his experiments were made upon the young shoots of the potato and not upon leaves, the result obtained, in causing the amyloplasts of the plant to form starch under the influence of various carbohydrates, is interesting, as confirming what we have already said as to the identity of the starch-forming functions of the chloroplasts and the colourless amyloplasts of the plant.

In 1885, Schimper (35) published a lengthy paper dealing with the formation and translocation of starch in leaves. Confirming in the first place Sachs's observations on the disappearance of starch from leaves placed in the dark, he endeavours, chiefly by observations on *Impatiens* and *Hydrocharis Morsus-ranæ*, to follow out the processes of starch dissolution and the translocation of the products within the leaf. He employs almost exclusively microchemical methods based on the use of iodised chloral hydrate and Fehling's solution, and although his paper contains many interesting and valuable observations on the distribution of starch in the leaf, and indications of the channels through which its products of solution pass out of the leaf, it may be permitted to doubt whether the further employment of such microchemical, to the exclusion of chemical, methods is likely to materially advance our knowledge of the physiological processes of plant-life as evidenced in the leaf.

In 1890, Saposchnikoff (46), in an interesting and suggestive paper, which apparently must be considered only as a preliminary communication, attempted amongst other things to show by experimental methods the quantitative dependence of the carbohydrates of the leaf upon assimilation. He used the half-leaf method, applying to the one half Timiriazeff's delicate eudiometric process (33) for determining the amount of CO_2 decomposed in assimilation; and to the other half Faulenbach's method for the determination of the total carbo-

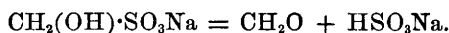
hydrates simultaneously formed. Faulenbach's method is not described, nor is any reference given to it, but as far as we have been able to discover, the process consists in previously treating the extract or material under experiment with malt-extract to bring the starch into solution, and then hydrolysing the starch-products and the other soluble carbohydrates down to the dextrose point with dilute HCl. This is a method which will no doubt approximately give the total assimilated carbohydrates, but it is manifest that it is impossible in this way to differentiate the sugars from starch or from each other. The general conclusions drawn by Saposchnikoff are: (1) the loss of carbohydrate from a cut leaf is much less than that from a leaf still attached to the plant; (2) the smaller the number of leaves on a plant the more rapidly, *cæteris paribus*, does depletion go on in the leaf; (3) the rapidity of translocation of carbohydrates is dependent upon the rate at which they are used up by the growing parts of the plant; (4) we know nothing with certainty of the form in which the carbohydrate wanders out of the leaf—apparently it is as *glucose*; (5) the plant-cells appear to be a self-regulating apparatus, re-forming starch out of the sugars when an excess of these are present, and re-dissolving the starch when their tissues are in want of soluble carbohydrates; (6) the clearer the sky and the more rapid the assimilation the greater is the production of carbohydrate; (7) accumulation of carbohydrates in leaves hinders the further production of those bodies, and, conversely, the quicker the carbohydrates are carried away from the leaf the better the leaf functions as regards assimilation; (8) there is more CO_2 decomposed during assimilation than is accounted for by the supposition that the whole of the carbon goes to form carbohydrate; consequently it is concluded that other substances are produced in the leaf, perhaps albuminoids.

In the same year in which Saposchnikoff published the paper last referred to, Timiriazeff (47), by the projection of the solar spectrum on a green leaf, demonstrated in a very beautiful and conclusive manner the fact that only those rays which are absorbed by green chlorophyll can bring about assimilation of starch within the chlorophyll corpuscles; as, however, this aspect of the subject does not immediately concern us in this enquiry, we merely make a passing reference to the paper, and also to the equally beautiful research made at an earlier date by the same author (33), dealing with the general functions of chlorophyll.

A series of experiments on the production of starch in plants supplied with ready-formed organic compounds was described by Acton in 1889 (48). His experiments were made by supplying the culture fluid to cut branches, to the roots of the plants, and to the surface of leaves. Before applying the culture fluids the plants were

depleted of their starch, this being in most cases brought about by placing them in an atmosphere absolutely free from CO_2 . The conclusions drawn from numerous experiments are that green plants cannot normally obtain carbon from any ready-formed substances except carbohydrates, or bodies closely related to them. An interesting fact is also established that a compound may be a source of carbon when supplied to the leaves, but not when supplied to the roots, and *vice versâ*. There was complete failure to establish starch-formation from aldehydes or their derivatives.

Opposed to these last-mentioned results are those of Bokorny (40), to which we must now refer. Bokorny's earlier attempt to produce starch in the green cells of *Spirogyra* directly from formaldehyde had been rendered abortive owing to the very poisonous nature of that substance, but he had obtained abundant evidence of starch-formation being induced in the plant-cells by a 1 per cent. solution of methylal. As, however, there was a doubt in this case whether the starch had been formed from formaldehyde or from methyl alcohol, Bokorny in 1891 approached the subject again (54), using as a nutrient a dilute solution of sodium oxymethylsulphonate, $\text{CH}_2(\text{OH})\cdot\text{SO}_3\text{Na}$, which on warming with water splits up into formaldehyde and acid sodium sulphite,



Dipotassium or disodium phosphate is added to prevent the unfavourable action of the acid sodium sulphite upon the plant-cells.

In dilute solutions of 1 per 1000 to 1 per cent., to which a mineral nutrient had also been added, it was found that *Spirogyra majuscula* readily formed starch, under conditions which precluded any possibility of the carbohydrate being formed from anything but formaldehyde. Bokorny rightly looks upon these results as being confirmatory of Baeyer's views on the chemical processes involved in assimilation (63). The recent work of E. Fischer on the synthesis of the sugars has still further strengthened this hypothesis.

Having considered the present state of our knowledge with regard to the occurrence and formation of the starch of leaves, it now becomes necessary to enquire how much is known of the causes which bring about the dissolution and translocation of this starch temporarily stored up in the leaf tissue.

We have already seen that there is a striking analogy in structure, and under some conditions also in function, between the colourless amyloplasts of plant-cells and the green chloroplasts. That this is more than an analogy has been shown over and over again by different observers, who have noted the transformation, under certain condi-

tions, of the non-assimilating amylopastas into assimilating chloroplasts, and *vice versâ*; whilst it has also been shown that truly assimilating chloroplasts can form starch within their substance at the expense of ready-formed carbohydrates when these are supplied artificially to the plant-cells. This being the case, it seems almost certain that the mechanism involved in the redissolution of the starch formed in the two kinds of plastids must be identical, and it, therefore, becomes necessary to enquire into what is known about the chemical phenomena attending the dissolution of starch temporarily stored in any part of the plant, as it is in the highest degree probable that the same laws regulate also the dissolution and translocation of the autochthonous starch of foliage leaves.

Although several observers, guided by the phenomena of germinating starch-containing seeds, have expressed an opinion that all transitory or reserve-starch of plants owes its dissolution to the occurrence of *diastase* in the plant, we are not aware that any serious attempt was made to prove this experimentally until 1877, when Kosmann (15) stated that by precipitation of aqueous infusions of algæ, lichens, moss, and certain fungi with alcohol, he had obtained a substance which had in all cases a distinct action on starch-paste, and even in some cases on unaltered starch. No precaution seems to have been taken to prevent the growth of organisms in the extracts, nor to differentiate the action of such organisms from that of a true enzyme.

In the following year, Baranetzky (16) published the results of very numerous experiments he had made to ascertain the presence of starch-dissolving ferments in various parts of plants. In addition to his experiments on germinating seeds, Baranetzky extended his observations to starch-containing bulbs, and also to the stems and leaves of many plants. He also studied the mode of attack on the solid starch-granules and the phenomena attending their dissolution, both within the tissues of the plant and under the right conditions *in vitro*.

The usual method of experiment was to make aqueous extracts of the ground-up tissue, and to precipitate these with alcohol. The precipitate was redissolved in a little water, and its action tried on a 1 per cent. starch-paste at the ordinary temperature. If the starch-paste became limpid, the vegetable tissue was considered to contain *diastase*.

In this way, or by using the more active extracts direct, *diastase* was found to be present in the stems and leaves of various plants. Baranetzky does not doubt that the starch-dissolving enzyme is instrumental in bringing about within the plant the conversion of the starch-granule into soluble carbohydrate. As the extract of the

plant-tissue, or preparations from it, have only a feeble starch-hydrolysing action, it is concluded that the quantity of enzyme existing in the plant-organs at any one time is always very small, and that there is compensation for this in the living plant by a constant reproduction of diastase by the plant-cell in sufficient amount to supply all the needs for starch-translocation, but that this reproduction does not proceed sufficiently rapidly for an accumulation of enzyme to take place.

In 1883, Böhm (30), whilst drawing attention to the fact that starch-dissolution in the plant has generally been attributed to the presence and action of soluble ferments, states as a somewhat unexpected result that leaves which have been frozen and then thawed are not depleted of their starch, nor are leaves which have been surrounded by an atmosphere of hydrogen. He believes, nevertheless, that the dissolution of starch in the leaf is brought about by diastase, holding much the same views as those previously expressed by Baranetzky, viz., that the ferment is used up almost as fast as it is produced, and that consequently the amount found at any one time is very small.

Brasse, in 1884 (31), made an examination of the leaves of *potato*, *dahlia*, *Jerusalem artichoke*, *maize*, *beet*, *tobacco*, and *Ricinus*.

He precipitated an "amylase" from the aqueous extracts by means of alcohol, just as Kosmann and Baranetzky had done before him, but in the subsequent action of this amylase upon starch he used chloroform to inhibit the growth of organisms, and measured the rate of the starch-transformation by the cupric-reducing power of the solution. These experiments are really the first on record which to our mind conclusively prove the existence of diastase in foliage leaves.

Sachs, in his paper of 1884 (32), does not add any new facts to this part of the subject, except by showing that the starch of the chlorophyll-granules can be dissolved by malt-extract. He leaves it an open question whether in the living plant the starch is dissolved by some power possessed by the chlorophyll-granule, or whether it owes its disappearance to the action of soluble ferments.

A little later Cuboni (36) expressed the opinion that the phenomena cannot be explained entirely by one hypothesis or the other.

Schimper, in 1885 (35), described some experiments he made with extracts of leaves, all of which were found to be feebly diastatic, the criterion of activity being the liquefaction of starch-paste by the extracts. Having a suspicion that the peculiarity of *Allium Cepa* in not forming starch within its chlorophyll-granules might be due to the presence of a large amount of diastase in the plant inhibiting the formation of solid starch, he made some comparative experiments

with the leaves of *Allium* and those of plants like *Tropæolum* and *Euphorbia Peplus*, which forms abundance of starch in the leaf. The expected correlation between the occurrence of starch and diastase was not found to exist, since the starch-free *Allium* leaves were much less diastatically active than the leaves of *Tropæolum* or *Euphorbia*.

Little or nothing further seems to have been done to advance this particular branch of the subject we are considering until 1890, when three memoirs appeared having a more or less direct bearing upon the mechanism of starch-dissolution in the plant. We will consider them in the order of their publication.

In a paper dealing with the germination of some of the Gramineæ (44), we had occasion to draw attention to the fact that during the germination of barley, the diastase which is found in the growing parts of the young plant, the radicles and plumula, differs very materially in certain ways from the diastase which is secreted by the epithelial layer external to the scutellum. Whilst this last-mentioned diastase is capable of readily liquefying starch-paste, and of actively pitting and eroding the solid starch-granule, the former has but little power of acting either on starch-paste or on the solid starch-granule, although it can energetically hydrolyse soluble-starch. We pointed out that the first-mentioned variety of diastase corresponds in properties with those of the comparatively feeble enzyme which has from time to time been obtained from various tissues of plants, and as it seemed correlated with the disappearance of starch in such tissues, we spoke of it as "diastase of translocation," whilst the more energetic or active form, which is secreted by the embryo during germination, we referred to as "diastase of secretion" (*loc. cit.*, p. 509). The diastatic activity of the growing parts of the embryonic barley-plant was determined, and also that of ungerminated grain, the diastase present in the latter case being regarded as the unused residue of the translocation-diastase originally produced by the parenchymatous cells of the endosperm during their development.

The second paper is one by J. Wortmann (45), and treats of the occurrence and significance of the diastatic enzyme of plants.

As this is an important memoir, and one to which we shall have occasion to make frequent and critical reference, it will be necessary to consider it somewhat fully.

After referring to the almost universal belief that diastase occurs in all parts of plants, and that where starch exists in living plants its dissolution is brought about by this diastase, Wortmann expresses his dissent from these views, basing his objection in the first place upon the very feeble diastatic action of leaf-extract in such experiments as those of Schimper, and in the second place upon the very small amount of enzyme they indicate as compared with the actual

amount of starch dissolved in a given time in the living leaf, an amount which Sachs has shown may reach 1 gram per square metre in a single hour of the night. He also objects to the iodine reaction as a sole criterion of starch-dissolution, maintaining that there ought always to be a microscopic control of the corrosion of the starch-granules.

Wortmann also insists upon the time occupied in making the plant-extracts being reduced within narrow limits, so as to avoid the appearance of organisms, some of which are themselves producers of diastatic enzymes.

After describing his experiments on germinated and ungerminated seeds of various kinds, Wortmann proceeds to an examination of leaves. The extracts were made by shredding and pounding the leaves with a little water for from two to six hours.

Starch-paste of 0.5 per cent. was used as the reagent, and also unaltered wheat-starch. In all, the leaves of 32 different species of plants were examined in this way for diastase. In 27 of these cases no diastase was found in the extracts, and in the remaining five only traces of a starch-dissolving enzyme were discovered. From these experiments Wortmann draws the conclusion that diastase is either altogether absent from assimilating leaves, or occurs in such small quantities that it cannot have anything to do with the translocation of starch. This, in his opinion, is also further shown by the fact that when diastase does occur in the leaf, it is of such a nature, and so feeble in its action, that, unlike the diastase of germinating seeds, it is unable to exert any action on the solid starch-granule, a property which would certainly be required if its presence had any physiological importance. It is, therefore, concluded that dissolution of starch in the leaf is brought about *directly* by the protoplasm, and that no agent is present in sufficient quantity for bringing about starch-dissolution independently of the protoplasm of the leaf-cells.

In further proof of this proposition, Wortmann placed fragments of the leaf of a *Helianthus* in a moist place in the dark, and found that no disappearance of starch took place in the tissue. When starch-containing leaves are placed in an atmosphere free from oxygen, the activity of the chloroplasts is suppressed, but not the activity of any diastase which may be present, yet when starch-containing leaves still attached to the plant were surrounded with an atmosphere of CO_2 in a dark room, no disappearance of starch took place, although other leaves of the same plant which were freely exposed to the air became comparatively depleted of their starch in 20 hours.

The experiments were repeated by Wortmann with certain variations fully described in his paper, and all pointed to the same general

result, that when the vital functions of the protoplasm are arrested by depriving the cells of oxygen, no disappearance of starch takes place from the chloroplasts.

Apart from the special cases of the dissolution of starch in germinating seeds, Wortmann expresses a very strong opinion that diastase either takes no part at all in the solution of starch within the plant or but a very insignificant one, and that this dissolution is brought about by the direct action of the living protoplasm.

The third memoir of the year 1890 which requires special reference is by G. Krabbe (50). It is a long and elaborate paper dealing with the action of the diastatic ferment on the starch-granule, both within and without the plant.

A great number of experiments and observations were made upon various plants and their contained starches, and the conclusion is drawn that the visible mode of dissolution of the starch-granule within the plant corresponds to the mode observed when the starch is digested with solutions of diastase outside the plant. The dissolution of the starch of the plant-cell is not brought about by micro-organisms, as is asserted by Marcano and by Wigand (42), nor is it due directly to the action of the living protoplasm, but is produced by an amylolytic enzyme which is probably a split-body of protoplasm. As regards the wandering of diastase in plants, this, doubtless, cannot take place in the form in which it hydrolyses starch, owing to its great indiffusibility. If it wanders at all from cell to cell, Krabbe is of the opinion that it must undergo some chemical change and be again restored to its active form at the spot where it is required. It is, however, by no means certain that diastase ever is transported from one part of the plant to another, but it is far more likely that it only comes into existence in the place where it is wanted.

In 1891, S. H. Vines (55) published the results of an investigation he had made with a view to ascertain how far Wortmann is correct in his statement that green leaves contain little or no diastase. Vines objects to Wortmann's method of experiment, especially to his using the *filtrates* of the leaf extracts, as he has found that the merely strained and still turbid extracts are much more active diastatically than the clear filtrates. He also thinks that the non-disappearance of starch from leaves, when the leaf-cell is deprived of oxygen, as described by Wortmann, is capable of being explained on the assumption that in the absence of free oxygen protoplasm is unable to secrete the diastatic ferment. Results are given showing the amount of sugar, calculated as dextrose, formed by the action of 50 c.c. of leaf-extract on starch-paste. The cupric-reducing body so prepared from starch was shown to be a fermentable sugar, capable of yielding

a phenylhydrazine compound, but it is concluded that this sugar is not maltose, as it is said to possess no optical activity.

The final conclusion at which Vines arrives is that although the amount of ferment which can be extracted from the leaves at any given moment is small, yet this ferment is, doubtless, being constantly secreted, so that the total amount produced during a night may suffice to effect the observed conversion of starch into sugar.

The last paper which we shall have to notice in connection with the occurrence of diastase in plants is a recent note by A. Prunet (61) on the mechanism of starch-dissolution in the plant. It is an attempt to prove experimentally that a real relation exists between the production of diastase and the dissolution of starch. He had observed, during the sprouting of the tuber of the potato, that the buds nearer the summit develop more rapidly than those nearer the base. It seemed reasonable to suppose, therefore, that at the commencement of growth the transformation of the reserve-starch of the tuber would take place more rapidly near the summit of the tuber than near the base, and that if diastase is really the agent of dissolution of the starch, this would also be found near the actively growing parts.

This was verified experimentally. Prunet estimated the solution-products of the starch (dextrin and sugar) as glucose, after boiling the aqueous extracts of the tissue with acid. The diastase was determined by precipitating the extracts with alcohol and determining the rapidity with which a solution of the precipitate would act on starch-paste, using iodine as an indicator.

The results are contradictory to those of Wortmann, and indicate that there is really a relation between the distribution of diastase and the solution of starch in the sprouting potato, and that a relation also exists between the appearance of diastase and the commencement of growth in the tubers.

3. *The Starch of the Leaf, its Determination, and the Proportion it bears to the Total Products of Assimilation.*

The iodine-method devised by Sachs in 1884 (32) for the macroscopical detection of starch in the leaf is undoubtedly very useful when the difference in the amount of starch in the two compared pieces of leaf is considerable. The rapidity with which the process can be carried out is much in its favour, but if the actual amount of starch in the mesophyll is small, the coloration of the tissue by the iodide of starch is often completely masked, or differences in amount of starch, which may be very appreciable when some other method is employed, are inappreciable with the iodine-test.

These objections do not apply to a method originally devised by A. Meyer, and subsequently perfected by Schimper. In this case the leaf is first completely decolorised with alcohol, but is not, as by Sachs' method, previously treated with boiling water. It is then immersed in a concentrated solution of chloral hydrate, to which a little iodine has been added. Under these conditions the leaf-tissue becomes very transparent, and the swollen and iodine-stained granules of starch can be examined *in situ* with the aid of the microscope.

Although requiring much more time than Sachs' iodine-test, this method commends itself for the certainty with which even very minute traces of starch can be detected, for the accuracy with which small differences in the amount of starch can be appreciated in comparative experiments, and for the ease with which the exact distribution of the starch in the various parts of the leaf-tissue can be ascertained.

The observations of Sachs which led him to conclude that starch-production in the chlorophyll-granule is dependent upon assimilation have been so frequently repeated and confirmed by others, that it is unnecessary to record any of our own experiments in this direction, all of which are confirmatory of the exactitude of Sachs' statements on these points. We have also on many occasions repeated successfully the experiments of Böhm and of A. Meyer upon the nutrition of leaves with solutions of carbohydrates, and can fully confirm their statements that the very chloroplasts which, under the ordinary conditions of the living plant, form autochthonous starch by the processes of assimilation, can also elaborate a similar starch from nutritive solutions of certain sugars. This is a fact of immense importance for the study of the assimilative processes, and is one to which we think far too little attention has been given by vegetable physiologists. Although at first sight it may appear that there is a radical difference between the formation of starch in the chloroplasts of an actively assimilating plant and the formation of starch in those same chloroplasts from a solution of sugar, such is not really the case. It is perfectly true, as pointed out by Sachs, that starch is the first *visible* product of assimilation, yet there can be little doubt (as was, in fact, anticipated by Sachs himself) that between the inorganic substances entering into the first chemical process of assimilation and the starch, there is a whole series of substances of the sugar class, and that it is from the last members of this series that the chloroplasts, under normal conditions, elaborate their starch. Both under the natural conditions of assimilation and the artificial conditions of nutrition with sugar solutions, the chloroplasts form their included starch from antecedent sugar.

The view originally put forward by Baeyer, that these sugars of

the assimilating organs owe their origin to the polymerisation of formaldehyde, has received remarkable experimental support from the direct observations of Bokorny already alluded to (54), and from the recent work of Emil Fischer on the synthesis of the sugars.

We have already referred (p. 610) to the description given by Sachs, in his 1884 paper, of the method he employed for determining the amount of starch produced or lost by foliage leaves, a method based upon the change in dry weight of accurately known areas of leaf cut from either side of the mid-rib. If we examine this method, however, we see that it cannot give us the actual amount of *starch* assimilated within a certain period, but that it really measures the *total assimilated products* which enter or leave the leaf during a given time. Sachs speaks of it throughout his paper as a gain or loss of *starch*, because he assumes that all the products of assimilation in the leaves of most plants pass at one time of their history through the form of starch in the chlorophyll-granules. We have, however, no proof that starch is an *essential* link in the chain of chemical products, beginning with the inorganic materials and ending with the form in which the products leave the leaf. That much of the first products of assimilation does pass through the starch stage under ordinary conditions there can be no doubt, especially when the process of assimilation is active; but no sufficient experimental proof, or, in fact, proof of any kind, has been given of that constant and extremely rapid flux of starch within the chlorophyll-granule which must occur if the assumption of Sachs is correct. The behaviour of the chloroplasts of the assimilating cells when the leaf is immersed in nutritive sugar-solutions, as in the experiments of Böhm and Meyer, seems to indicate that they, like the colourless amyloplasts, only form starch, either when the supply of formative carbohydrates is in excess of the metabolic and translocating power of the cell in which they are contained, or when the sugar-solution has reached a certain degree of concentration. On this view, which we think is in accord with all the facts, the starch of the chlorophyll-granule is as truly a transitory reserve-starch as that of any other part of the plant,* a reserve which is drawn upon after the intensity of the light has been so far diminished as to materially retard assimilation.

* Because Sachs found that the starch disappeared from the chlorophyll-granules of a fully insulated leaf when the plant was placed in an atmosphere free from CO_2 , he concluded that starch-dissolution, as well as starch-formation, is continuously going on in a plant exposed in daylight to ordinary conditions. Under the artificial conditions of this experiment, of course, no assimilation was possible, and the starch stored in the chlorophyll-granules was necessarily drawn upon for the requirements of the plant, exactly as would have been the case in the dark. The experiment only proves that dissolution of starch is not inhibited by light.

Nevertheless this quantitative method of Sachs is a valuable one if we bear in mind its limitations, *i.e.*, that it does not indicate the amount of *starch* entering or leaving the leaf, but the amount of assimilation products, leaving for the moment the nature of those products for further consideration.

The conditions for cutting the rectangular pieces of leaf used in the experiment are carefully laid down by Sachs, and they are comparatively easy to fulfil in the case of large leaves, but we were met at the outset by this difficulty, that we did not know how far the observed variations in weight were likely to be due to accidental variations in the thickness and density of different parts of the mesophyll and veins, and how far to real accumulation or loss of assimilated material. Upon this important question of the probable error of the method Sachs is quite silent, although it is reasonable to suppose that so able an experimenter had fully satisfied himself on this point.

In order to test the error likely to occur from the above-mentioned structural differences in the leaf, we made the following experiments.

Seven leaves of *Helianthus annuus* were taken, and from these, with the aid of glass templates, one of which was 10 cm. square, and the other 10 cm. by 5 cm., equal areas of leaf were cut out from each of the right- and left-hand halves. The leaf area taken in each case amounted to exactly 800 sq. cm. The pieces on either side of the leaf were cut out symmetrically, the larger veins being avoided as far as possible, and pains were taken to include about the same proportion of the smaller veins on one side as on the other. The equal areas of leaf were then quickly killed with steam, and dried in the water-oven. On weighing, the following results were obtained.

1. 800 sq. cm. of right-hand halves, 3·2430 grams.
2. 800 „ „ left-hand „ 3·2772 „

These correspond to the following weights per square metre.

1. 40·53 grams.
2. 40·96 „

These results, differing to the extent of less than 0·5 gram per square metre of leaf-surface, or about 1 per cent., may be considered satisfactory, as, in the case of *Helianthus*, Sachs found increases and decreases of weight, under varying conditions of assimilation and depletion, equal to from 9 to 11 grams per square metre, numbers very far in excess of anything attributable to variation in weight of different parts of the leaf.*

* It must be borne in mind, however, that leaves vary considerably amongst themselves in weight per unit of area, and in order to make comparisons as

The following experiments, carried out on the lines laid down by Sachs, confirm his statement that assimilation goes on under ordinary conditions sufficiently rapidly to cause a very sensible increase in weight of a given area of leaf.

At 8 A.M., on August 18, 12 healthy leaves of *Helianthus annuus* were cut from the plant. One half of each leaf was at once divided into rectangular pieces of known area, in the manner already described, taking care not to injure the mid-rib; the area of leaf so obtained amounted to 1650 sq. cm., and the dry weight was 6.7674 grams. The other half of each leaf was placed with its petiole immersed in water, and was exposed to daylight in the open, the day being dull, without sunshine.

In this latter case, the products of assimilation would necessarily accumulate in the tissues of the leaf.

At 5 P.M., from these insulated halves, 2100 sq. cm. of leaf were cut, and these, when dried, weighed 10.4750 grams. Calculating the results out for the square metre of leaf-surface in each case, we have—

Weight of 1 sq. m. of leaf at 8 A.M.	41.01 grams.
" " " after nine hours' insol- ation	49.88 "
Increase of weight per sq. m. due to assimilation.	8.87 "
Assimilation in 1 hour per square metre	0.985 "

An experiment was carried on simultaneously with the above, in which the second half of each leaf remained *still attached to the plant* during the nine hours' insolation, so that the products of assimilation, instead of accumulating in the leaf, as in the last experiment with plucked leaves, were constantly passing out through the petiole into the stem of the plant.

The dry weight of 1425 sq. cm. at the commencement was found to be 7.5152 grams, whilst after nine hours' insolation of the other half-leaves which had remained attached to the plant, the dry weight of 1600 sq. cm. was found to be 8.5118 grams.

Calculating for equal areas, we have—

Weight of 1 sq. m. of leaf at 8 A.M.	52.73 grams.
" " " after nine hours' insol- ation on plant	53.19 "
Increase.	0.46 "

accurately as those just described, it is necessary to take approximately equal areas from each side of every individual leaf.

There has been practically no gain in weight of the leaf under these conditions, for the difference falls within the error of the method.

Since the conditions and time of insolation were exactly the same in this case as in the last, the amount of assimilated products formed must have been the same, *i.e.*, about 9 grams per square metre, but this gain has been obscured by an equal loss, due to the passage of the assimilated products out of the mesophyll into the principal ribs, petiole, and stem. Assimilation and depletion must consequently have proceeded at the rate of a little less than 1 gram per square metre per hour, which is almost exactly the rate of depletion which Sachs found for the *Helianthus* leaf during a summer night. The rate of assimilation was low in this experiment, owing to the cloudy state of the weather. Under favourable conditions of sunlight, assimilation proceeds much more rapidly than the products can pass out of the leaf, as is well shown in many of the experiments described by Sachs, and also by the following experiment, carried out under more favourable conditions of weather.*

On August 23, a bright warm day, with the sun occasionally obscured by cloud, the half-leaf experiment was made upon leaves of *Helianthus annuus*. At 5 A.M., 1525 sq. cm. were cut from one side of the healthy leaves, and the dry weight was determined as usual. At 5 P.M., an approximate equal area—1550 sq. cm.—was cut symmetrically from the other halves of the leaves, which had been left attached to the plant during 12 hours of daylight.

The dry weights in both cases were calculated from the square metre, and were as follows.

Weight of 1 sq. m. of leaf at 5 A.M.....	50·006 grams.
" " " at 5 P.M.....	58·566 "
<hr/>	
Increase in weight per sq. m. during 12 hours' insolation on the plant.....	8·560 "
*Excess of assimilation over depletion per sq. m. per hour	0·713 "

* The comparative rarity of bright cloudless days in the Midland Counties during the autumn of 1891 and the summer and autumn of 1892 was a frequent bar to the progress of this investigation, and much patience and watchfulness were required to avail ourselves of the by no means frequent opportunities of successful experiment. Our English climate is not one which is favourable at any time to a research of this nature, owing to the rarity of days of continuous sunshine during which assimilation is at a maximum.

† We have taken no account in these experiments of the loss due to the *respiration* of the leaf-cells.

The experiments of Weber indicate that in the case of *Helianthus* leaf the loss of

Concurrently with the above experiment, the half-leaf method was applied to other leaves of *Helianthus annuus*, which in this case were separated from the plant and placed with their petioles in water from 5 A.M. to 5 P.M. under as nearly as possible the same conditions of insolation. The following are the results of weighing:—

Weight of 1 sq. m. of leaf at 5 A.M.....	50.350	grams.
" " " after plucking and in-		
solating from 5 A.M. to 5 P.M.....	62.376	,,
	<hr/>	
Increase per square metre	12.026	,,
Assimilation per square metre per hour	1.00	,,

All our results so far have been strikingly confirmatory of the accuracy of Sachs' experiments and of the general applicability, within certain narrow limits, of his method of determining the rate of assimilation and depletion of leaves by the change in weight of the leaf-lamina.

We have now to enquire how much of the assimilated products of the mesophyll is really present at any one time in the form of starch.

We have found it possible to estimate the starch of leaves with great exactness by a method which is substantially identical with one described by O'Sullivan (Trans., 1884, 45, 1) for the determination of the starch of grain.

It is essential in the first instance to obtain the leaves in a perfectly dry state, and in order to do this it is necessary to adopt certain precautions, otherwise the amount of starch found will be very much less than that which the leaf contains at the time of plucking.

When a starch-containing leaf is slowly dried at a temperature of from 30° to 40°, the application of the iodised chloral hydrate test from time to time indicates a *gradual but well-marked diminution in the starch* up to the time of the complete expulsion of moisture. Under what influences this disappearance of starch takes place, we shall consider at a later stage of this enquiry.

In order to avoid this loss of starch, we must either kill the leaf, by immersing it for a short time in air containing chloroform vapour, or we must dry the leaf rapidly at a temperature of 75–80°. If the leaves have been previously immersed in alcohol, no special precaution is requisite in drying.

weight due to this cause does not exceed about 0.05 gram per square metre per hour, an amount so small compared with other sources of error inherent to the method that it may safely be neglected. We shall have occasion to revert to this question of respiration of the leaf later on in this paper, when considering the sugars of the leaf.

The dried leaf is then finely powdered and thoroughly extracted with ether in an extraction-apparatus to remove chlorophyll, fats, &c. When the ether passes through the material without taking up any colour, the powdered leaves are dried and a definite weight taken for the starch determination. This weighed quantity (generally about 10 grams will suffice) is placed in a flask and digested with alcohol of about 80 per cent. for 24 hours at 40°; the alcohol is then poured off and the extraction repeated. After the second extraction, the substance is repeatedly washed by decantation with warm alcohol until the washings are free from colour. Experience has shown us that subsequent extraction of the leaf with cold water, prior to the starch-determination, is unnecessary.*

The residue from the alcohol treatment, after drying, is mixed with a little warm water, and the mixture heated in a bath of boiling water for a little time to complete gelatinisation of the starch; it is then cooled to 50°, a little prepared diastase is added, and the conversion of the starch is carried on at 50—55° for about two hours. The mixture is raised to the boiling point once more, the solid matter removed by filtration, the filtrate made up to a definite volume, and the optical activity and cupric-reducing power of the filtrate determined in the usual manner.

From these data it is easy to calculate the actual amount of maltose and dextrin produced by hydrolysis, and hence the amount of starch originally present in the leaf.

The following example will illustrate the working of the method, and will also serve to show the very concordant results which may be obtained.

A batch of leaves of *Tropæolum majus*, picked after a sunny day, was quickly dried in a steam oven. Two lots of exactly 10 grams each, A and B, were then taken, and these were extracted successively by ether and alcohol in the manner described.

Sample A, after treatment with boiling water, was cooled down to 50° and digested at that temperature with a little diastase for two hours.

* In the somewhat slow process of washing with water, as recommended by O'Sullivan, there is always a great risk of loss of starch if the material is at all diastatic, especially of those starch-granules which are broken and more or less disintegrated. In the case of germinated grain, a loss of starch in this way is certain to occur if special precautions are not taken. If such washing is absolutely necessary for the separation of amylans, we have usually employed a dilute solution of salicylic acid for the first treatment, until the diastase is removed. A much better plan, however, is to treat the material previously with *boiling* alcohol of 80 per cent. This completely paralyses any subsequent action of the diastase without gelatinising the starch. (T. C. Day. Private communication.)

After complete conversion, the mixture was filtered and the filtrate and washings made up to 144 c.c.

Optical activity in 100 mm. tube, 1.9 divisions.

100 c.c. of the filtrate reduced 0.532 gram CuO.

0.532 gram CuO is equivalent to 0.395 gram maltose.

This amount of maltose in the 100 mm. tube will rotate the polarised ray through 1.54 divisions of the scale; therefore $1.9 - 1.54 = 0.36$ division due to dextrin. This corresponds to 0.064 gram of dextrin.

We find, therefore, that

100 c.c. of the filtrate contain 0.395 gram maltose.

” ” ” ” 0.064 ” dextrin.

Since the total volume of the filtrate is 144 c.c., the 10 grams of leaf have yielded

Maltose 0·5688 gram.

Dextrin	0.0922	„
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But 0.5688 gram maltose is equivalent to 0.5486 gram starch,
and 0.0922 „ dextrin „ 0.0922 „ „

Therefore 10 grams of leaf contain 0.6408 " "

i.e., 6·408 per cent.

In the duplicate sample B, the same procedure was adopted with the exception that the starch was hydrolysed with a small quantity of a cold-water infusion of malt instead of prepared diastase. The numbers given below are all corrected for the optical activity and reducing power of this small quantity of malt-extract.

Volume of filtrate, 144 c.c.

Optical activity in 100 mm. tube, 1.82 divisions.

100 c.c. reduce 0.646 gram CuO .

0.646 CuO equals 0.480 gram maltose.

This amount of maltose will give an optical activity in the 100 mm. tube of 1.87 divisions, against 1.82 actually observed, so that the conversion has been carried completely down to maltose.

On calculating out the results in the same manner as the last example, we find that from 10 grams of the leaf we have obtained 0.6912 gram of maltose. This is equivalent to 0.6545 gram of starch.

Therefore 10 grams of leaf contain 0.6545 gram starch = 6.545 per cent.

In the two experiments we have obtained—

	Percentage of starch.
Experiment A	6.408
Experiment B	6.545

These results are fairly representative of the accuracy which can be attained by careful working.

Below we give some results of the examination of leaves of *Tropæolum majus* for starch by the diastase method. As we have ascertained that the weight of a square metre of the leaves of this *Tropæolum* weighs, on an average, about 27 grams, we have given the results both in actual percentages on the weight of the dry leaf and in grams of starch per square metre.

With the exception of 1, in which the leaves were plucked at 9 A.M., the experiments were made with leaves taken from the plant during the afternoon, but under variable conditions of weather.

	Percentage of starch on dry leaf.	Grams of starch per square metre (approximate).
1	2.926	0.790
2	6.380	1.722
3	7.440	2.008
4	4.930	1.330

By the aid of the diastase method, we have been able to determine with accuracy the amount of starch which disappears from the leaf in darkness within a certain time. The following are some of the results obtained :—

A large number of leaves of *Tropæolum* were picked at 5 P.M. after a warm day, when the iodine test indicated an abundance of leaf-starch. These leaves were divided into two portions, A and B. The leaves of A were dried at once in the steam-chamber, whilst those of B were placed in the dark for 63 hours with their petioles in water, and afterwards dried in the same manner. The iodine-test indicated a very marked, but by no means complete, disappearance of starch in B. On estimating the starch by the diastase method, we obtained the following numbers.

	Percentage of starch in dry leaf.	Grams of starch per square metre (approximate).
A. Leaves taken directly from plant ..	6.505	1.756
B. Leaves depleted in darkness for 63 hours.....	2.055	0.554

In two other series of experiments conducted on the same lines, we obtained results as follows.

	Percentage of starch in dry leaf.	Grams of starch per square metre.
C. Leaves taken after a fairly sunny day	3.693	0.997
D. The same leaves depleted in darkness for 24 hours	2.980	0.804
E. Leaves taken after bright warm day	5.425	1.464
F. Same leaves depleted in darkness for 24 hours	0.906	0.244

We have been able to ascertain with a close approximation to accuracy the amount of assimilated material which is accumulated by the leaves of *Tropæolum* during a summer's day. From 9 A.M. to 5 P.M. on August 12, a period of eight hours, 1 sq. m. of the leaves increased in weight 7.2 grams, showing an increase of assimilation over depletion of 0.9 gram per square metre per hour. If we compare this result with the determinations of starch in a square metre of *Tropæolum* leaf, we see that the actual amount of starch present at any one time in the leaf must represent only a small proportion of the material assimilated during a summer's day of sunshine.

This conclusion is even more strikingly shown by the following experiment on measured areas of the leaf of *Helianthus annuus*.

On August 23, the day being sunny and warm, with occasional cloud, definite areas were cut from one side of a number of healthy leaves at 5 A.M., an equal area being cut from the other halves, which were allowed to remain attached to the plant until 5 P.M.

Both the dry weight and the total amount of starch were determined in each series with the following results.

	Weight of 1 sq. m. of leaf. Grams.	Starch per cent.	Starch in grams per square metre.
At 5 A.M.	50.006	2.01	1.05
At 5 P.M.	58.566	4.19	2.45
Increase in 12 hours..	8.566	2.18	1.40
Increase in 1 hour ...	0.713	0.18	0.12

The actual amount of materials assimilated by the leaf in the 12 hours was found, by a parallel experiment on cut leaves, to amount to over 12 grams per square metre; but whilst this large amount of material has been produced in the assimilating cells, and passed on to the other parts of the leaf or stem, there is only an observed increase of starch during that period of 1.40 grams. If, therefore,

Sachs' idea is correct, that all the assimilated products pass through the form of starch, the formation and dissolution of that substance must take place at a most astonishing rate. As we have before said, there is no evidence whatsoever of starch being a necessary link between the sugars of assimilation and the sugars of translocation; it is far more probable that starch is only elaborated within the cell when the supply of nutriment is in excess of the cell requirements, and that most of the assimilated products never pass through the stage of starch at all.

4. *The Occurrence of Diastase in the Leaf.*

We have already referred to the conflicting views which are held on the question of the mechanism by which the starch of the leaf is dissolved preparatory to its translocation; and we have dwelt somewhat fully upon the objections recently made by Wortmann to the current opinion that this dissolution is brought about within the plant by *diastase*. To substantiate his case that the dissolution is independent of the action of diastase, Wortmann relies upon the following supposed facts:—(1.) The absence of diastase from most leaves, or its occurrence in very small quantities only. (2.) When diastase does occur it is of such a nature, and so feeble in its action, that, unlike the diastase of germinating grain, it cannot act upon the *solid starch-granule*. (3.) When the vital functions of the protoplasm are arrested by depriving it of oxygen, then no disappearance of starch goes on in the leaf.

As regards (1) the whole of our work points consistently to the inaccuracy of the statement.

So far from leaves containing, as a rule, little or no diastase, we have never found a single case where diastase was not present in sufficient quantity to transform far more starch than the leaf can ever contain at one time; in fact, we shall see that diastase is frequently present in leaves to an amount sufficient, under favourable circumstances, to hydrolyse many times more starch than the total dry weight of the leaf tissue itself.

The method which Wortmann employed for investigating the leaf-diastase was a very crude one, and we are not surprised it led him to erroneous conclusions. The absence of diastase from a clear filtrate obtained after merely digesting the crushed leaves with water for three or four hours is no sufficient proof that the leaf does not contain a starch-transforming enzyme; for it is well known to those who have spent much time in separating such enzymes from vegetable or animal tissue, that the protoplasm often parts with its enzyme with considerable difficulty, and that it is often almost impossible to obtain

from such a preparation a clear filtrate which has any hydrolysing action, although an energetic hydrolysis may be produced by contact with the tissue itself, or by the employment of a turbid filtrate containing the finely-divided tissue in suspension (*cf.* Brown and Heron, *Proc. Roy. Soc.*, 1880, 393).

At the outset of our investigation this was found to be the case with the leaf extracts, *i.e.*, that, although the clear, filtered extract of the leaf may possess but feeble diastatic action, the leaf tissue itself can readily hydrolyse starch. Vines also called attention to this fact in his paper of 1891 (55). It will be shown later on that the difficulty experienced in procuring a diastatic infusion of a leaf is also materially influenced by the presence of *tannin*, and that when *tannin* is present in considerable amount it is quite impossible to obtain an extract of the leaf with any starch-hydrolysing power, although by suitable means it may be possible to demonstrate the presence of a very considerable amount of diastase in the leaf itself.

It is only by *drying* the leaf that the full activity of the contained diastase can be appreciated. If a leaf is air-dried at about 40° to 50°, then finely powdered, and put under favourable conditions for hydrolysing starch, the hydrolytic action is very much greater than that of freshly pounded undried leaves. It can readily be shown that this increase of diastatic power is not due in any way to the indirect influence of micro-organisms on the leaf-tissue during the slow process of drying, but to the much more complete disintegration of the leaf and the readier manner in which the enzyme is separable from the killed and dried cell-protoplasm.

The result of a single experiment, which may be taken as typical, will show the comparative diastatic activity of a leaf-extract and of the leaf-tissue itself. The experiments were made by a method presently to be described, and in such a manner as to give strictly comparative results. The leaf used was that of the *Helianthus tuberosus*, and the extract was made by a prolonged action of the water upon the crushed and pounded fresh leaf, a little chloroform being added as an antiseptic. The following were the results obtained:—

Diastatic activity of 10 grams of the dried leaf of	
<i>Helianthus</i> acting by contact	3·78
Diastatic activity of the filtered extract of 10 grams	
of the same leaf.....	0·53

5. What is the Nature of the Products of Transformation of Starch by Leaf-diastase?

Although we should have good reason to conclude from analogy that the products of the hydrolysis of starch by leaf-diastase are identical with those obtained under similar conditions with the

diastase of germinated grain, we are not justified in taking this for granted, especially as Vines has recently stated that the sugar produced is not *maltose*.

In the first experiment, the leaves of *Tropæolum majus* were employed.

2·5 grams of the dried leaves, acting for seven days at 30° on 100 c.c. of a solution of soluble-starch, under the usual antiseptic conditions, gave, on analysis, the following numbers, after due correction was made for the opticity and reducing power of the leaf-extract itself.

Opticity in 100 mm. tube, 8·2 scale divisions.

CuO reduced by 100 c.c. of the solution, 2·813 grams.

This amount of CuO corresponds to 2·090 grams of maltose, which should give an opticity of 8·26 scale-divisions, against 8·2 actually observed. In this prolonged hydrolysis, we see that the starch has been entirely converted into *maltose*.

Another experiment with the leaf of *Pisum sativum* indicates the successive stages of the hydrolysis, and also serves to illustrate the great diastatic activity of the leaf of this plant.

0·5 gram of dried leaf in 500 c.c. of a solution of soluble-starch, at 35°.

Maltose formed per 100 c.c.

After 48 hours.....	1·381 grams.
„ 96 „	1·448 „
„ 6 days	1·606 „

A complete analysis of the solution was now made, with the usual corrections for the opticity and reducing power of the leaf-extract.

Grams of solids per 100 c.c. (3·86 divisor).. 2·425

Optical activity in 200 mm. tube 21·7 divisions.

100 c.c. reduce 2·1606 grams CuO.

These numbers correspond to

$$[\alpha]_{38.5} = 171.8^\circ, \\ \kappa_{38.5} \quad 40.41,$$

and indicate the following composition of the hydrolysed products:—

Maltose.....	66.24	} requiring $[\alpha]_{38.5}$ 172.2.
Dextrin.....	33.76	
	<hr/> 100.00	

These experiments, and many others, the details of which it is unnecessary to give, all show very clearly that the hydrolysis of

starch by leaf-diastase gives rise to the same products as those of malt-diastase. That *maltose* is the sugar which is formed was further proved beyond all doubt by its actual crystallisation from the alcoholic solution obtained by treating the products of starch hydrolysis with 80 per cent. alcohol, and by the preparation and identification of the maltoseazone.

In all the foregoing experiments there is no indication of the hydrolysis proceeding beyond the point of maltose; in other words, we have seen no evidence of the existence in the leaf of an enzyme capable of hydrolysing maltose to dextrose. In order to ascertain if any further action on maltose can be induced under favourable conditions, the following experiment was made.

100 c.c. of a solution containing 4.751 grams of maltose was digested at 30° with 2 grams of dried *Tropæolum* leaves which had been previously shown to be diastatically active. After due correction for the sugars pre-existent in the leaves, the following results were obtained:—

	Grams CuO reduced by 100 c.c.	Opticity in 100 mm. tube.
Before digestion.	6.686	18.8
After 7 days digestion at 30°	6.441	18.5

No sensible hydrolysis of the maltose had taken place, even after very prolonged action of the leaf-diastase.

In order to ascertain if any invertive action can be brought about by the leaf, a parallel series of experiments was made, using cane-sugar instead of maltose.

2 grams of the same *Tropæolum* leaves were digested at 30° with 100 c.c. of a solution containing 4.881 grams of cane-sugar under the usual antiseptic conditions. The digestion was continued for seven days.

	Grams CuO reduced by 100 c.c.	Opticity in 100 mm. tube.
Before inversion	—	9.4
After „ 	2.064	7.2

These numbers indicate a decided invertive action of the leaf equal to the production of 0.894 gram of invert-sugar, an amount equal to nearly 45 per cent. of the weight of the dry leaf taken.

Although this last experiment is highly suggestive of the leaf-tissue itself containing an invertive enzyme, it is not absolutely certain that this is the case, since it is quite possible that the invertase may be derived from minute quantities of fungi growing on and in the leaf. Further experiments upon this subject are desirable, especially in view of the fact, which will be referred to later on, that

the living cell of the leaf-parenchyma most certainly has the power of inverting the cane-sugar which occurs within it.

6. *Determination of the Diastatic Activity of Leaves.*

The determination of the actual amount of diastase present in any animal or vegetable tissue, and its expression in terms of absolute units, is of course impossible in the present state of our knowledge, and will doubtless remain impossible for a long time to come. For all practical purposes, however, it is sufficient to determine the enzyme *comparatively*, which can be done by measuring the amount of hydrolytic work a given amount of the tissue can perform under certain standard conditions and in a given time. Kjeldahl (*Résumé du Compte-rendu du Laboratoire de Carlsberg*, 1, 1879, 129) first laid down the foundation for accurate comparisons of enzyme-action in his investigation on the rate of starch-hydrolysis under well-defined conditions. He clearly showed, and the subsequent investigations of ourselves and others have confirmed the general accuracy of the statement, that providing a starch-transformation is not allowed to fall below a cupric-reducing power of ≈ 25 to 30 for the mixed products of hydrolysis, then, under identical conditions of time, temperature, and concentration, *the cupric-reducing power is proportional to the amount of diastase originally present*. This is Kjeldahl's "law of proportionality," and we have based upon it the following method for determining the diastase of foliage-leaves, a method which is almost identical with that already described by us in our paper on the germination of the Gramineæ (44).

A. 0.5 gram of the finely powdered, air-dried leaf is digested at 30° with 50 c.c. of a 2 per cent. solution of soluble-starch which has been previously prepared by the limited action of hydrochloric acid on starch according to the method of Lintner. The digestion of the mixture is carried on for exactly 48 hours, the danger of any appearance of microorganisms being averted by employing chloroform at the rate of 5 c.c. per litre of starch-solution.

B. A duplicate experiment is made by taking the same amount of leaf and starch-solution, *boiling* the mixture for a minute or two, and placing alongside the first solution. This solution B is used for correcting A for the cupric-reducing power and opticity of the sugars naturally contained in the leaf. It is an essential part of the process which must never be omitted, since leaves generally contain a very sensible and, in some cases, considerable amount of reducing sugars.

The difference between the cupric-reducing powers of A and B is a measure of the hydrolytic work done, and it becomes possible, when

2 x 2

the time and all other conditions remain constant, to compare the relative diastatic activities in this way if care is taken that the limits of Kjeldahl's law are not overstepped.

If, in the course of experiment, the reducing power of the solution exceeds κ 25—30 in the standard time, another experiment must be commenced, using a less quantity of leaf, the proportion of leaf to starch solution being decreased until the reducing power falls to below κ 30.*

The cupric reduction is calculated in all cases as *maltose*, and for purposes of comparison this is referred to 10 grams of the dry leaf; so that our numbers indicating the relative diastatic activity really represent *the number of grams of maltose which the diastase of 10 grams of leaf is able to produce from soluble-starch by hydrolysis in 48 hours, at a temperature of 30°*.

The following are examples of the application of the method, and fairly represent the degree of accuracy attainable.

Experiment on Air-dried Leaves of Tropæolum majus.

A. 0.5 gram of leaf in 50 c.c. of soluble-starch solution, digested for 48 hours at 30°.

10 c.c. of the filtered solution reduced 0.0918 gram CuO.

B. The same amount of leaf and of starch-solution *boiled* at once, and digested side by side with A.

10 c.c. reduced 0.0259 gram CuO.

Then $0.0918 - 0.0259 = 0.0659$ gram of CuO reduced by the products of starch-hydrolysis of 0.1 gram of leaf: therefore 10 grams of leaf acting upon a proportional quantity of soluble-starch will give a reduction of 6.59 grams CuO, which is equivalent to 4.90 grams of maltose. This is the maximum amount of maltose capable of being formed in 48 hours by the diastase of 10 grams of this particular leaf.†

In another experiment, made on another portion of the same sample of leaf under identical conditions, we found—

* That this mode of procedure, *i.e.*, reducing the amount of diastatic agent as compared with the volume of starch-solution, gives perfectly comparable results, is shown by an experiment quoted by Kjeldahl (*loc. cit.*, p. 142), in which he acted upon 25 c.c. and upon 100 c.c. respectively of the same starch-solution for the same time, with the same volume of malt-extract. He obtained the same amount of reduction in each case.

These results are also confirmed by our own experiments on lupin leaves, quoted in the text.

† Care must of course be taken in all such experiments to remove by boiling the small amount of chloroform used as the antiseptic, before determining the reducing power of the solutions, as chloroform has a marked reducing action on Fehling's solution.

Solution A 10 c.c. reduced 0.0948 gram CuO.

Solution B 10 c.c. „ 0.0280 „ „

Calculated out in the same manner as in the last experiment, we find that 4.960 grams of maltose have been formed by the diastase of 10 grams of leaf, as against 4.900 in the first determination.

The following experiment, carried out on the same general lines with the leaf of the lupine, shows that a high degree of accuracy can also be attained in comparative experiments when unequal quantities of leaf are taken, that is to say, when the proportion of leaf to starch is variable; but the “law of proportionality” must not of course be overstepped.

A. 0.25 gram of lupine leaf digested with 100 c.c. of soluble-starch solution (*i.e.* in ratio of 1:400) for 48 hours indicated, after the usual corrections were made, that 10 grams of the leaf under these conditions could produce 3.510 grams maltose.

B. 0.50 gram of leaf with 50 c.c. of soluble-starch solution (*i.e.* in ratio of 1:100) for 48 hours indicates 3.250 grams maltose as produced by 10 grams of the leaf.

It is not possible by this method to make comparisons of relative diastatic power unless the *times of digestion are equal*. Any convenient period of time can be used in such a series of experiments, but this period, having been once selected, should be adhered to, as it is not possible to correct for variations of time owing to the rapidity of transformation not bearing any simple relation to the amount of diastase. The following experiment indicates this very clearly:—

Time Experiment showing the Progress of Starch-hydrolysis brought about by Leaf of Tropæolum.

1 gram of dry leaf digested with 100 c.c. of soluble-starch solution at 30°.

After 24 hours 10 grams leaf produced 0.767 gram maltose.

„	48	„	„	„	1.015	„	„
„	72	„	„	„	1.123	„	„
„	5 days	„	„	„	1.357	„	„
„	7	„	„	„	1.582	„	„
„	16	„	„	„	1.930	„	„

Before proceeding to give the results of the examination of the foliage leaves of various plants for diastase, we must allude to a disturbing cause which is liable to enter into all these determinations, and which in some cases leads to a considerable *underrating* of the actual amount of diastase present.

We have already noted how very much less diastatic an aqueous extract of a given quantity of leaf is than the equivalent quantity of leaf-tissue acting upon the starch-solution by direct contact, and we have ascribed this difference, which is generally observable in the case of all vegetable and animal tissues, to the tenacity with which the cell-protoplasm holds fast to the very colloidal diastatic enzyme. Occasionally, however, we come across a case in which, although the leaf-tissue shows marked hydrolytic powers when acting by contact, its aqueous extracts, no matter how carefully prepared they may be, are absolutely free from diastase. This is notably the case with the strobiles of the hop. When the bracts which constitute the strobiles or "cones" of this plant are dried, and their diastatic action is measured in the usual manner, their contact action on starch is considerable, as is shown in the table on the next page. On the other hand, it was found impossible to produce an aqueous extract of the hop-strobiles possessing the slightest hydrolytic action, no matter how concentrated the extracts were, or how long the water was in contact with the hops.

On investigation, it was found that the *tannin* of the hop had prevented the diastase going into solution. So marked is this inhibiting action of the tannin upon diastase that an aqueous extract of hops, subsequently infused with a little malt, is found to be incapable of extracting any of the diastase of the malt. That this inhibitory action on the malt-diastase is due to tannin is clearly shown by the fact that the extract of hops loses this power of inhibition if its tannin is removed by shaking with a small amount of the raspings of raw hide. The experiments upon which these statements are based have been fully described by us in another paper dealing with the more technical aspects of the question. (See *Transactions of Inst. of Brewing*, 1893, 6, 94). They have an important bearing upon the present enquiry, as there is no doubt that the comparatively weak diastatic action of some leaves is due to the tannin they contain, and that neither the action of the leaf-extract nor that of the leaf-tissue itself gives a complete measure of the amount of active diastase present in the leaf at any one time. A little consideration will, however, show that in measuring the diastatic energy of a leaf which contains tannin in some of its cells we reduce the inhibitory influence of this tannin considerably by dilution in the process of measurement we have adopted, since the volume of starch-solution used is considerable as compared with the actual amount of dry leaf-tissue employed.

In the following table we give the results of the determination of diastase in the leaves of a number of plants by the method we have described.

The numbers show the relative diastatic activities of the dried

leaves acting by contact, and really represent the amount of maltose, expressed in grams, which 10 grams of the air-dried leaf will produce from soluble-starch by hydrolysis in 48 hours at 30°.

1. <i>Pisum sativum</i>	240·30
2. <i>Phaseolus multiflorus</i>	110·49
3. <i>Lathyrus odoratus</i>	100·37
4. <i>Lathyrus pratensis</i>	34·79
5. <i>Trifolium pratense</i>	89·66
6. <i>Trifolium ochroleucum</i>	56·21
7. <i>Vicia sativa</i>	79·55
8. <i>Vicia hirsuta</i>	53·23
9. <i>Lotus corniculatus</i>	19·48
10. <i>Lupinus</i> (? sp.)	3·51
11. Grass with clover	27·92
12. <i>Tropæolum majus</i>	4·90
13. " "	4·96
14. " "	8·29
15. " "	9·64
16. " "	7·43
17. " "	4·25
18. " "	3·91
19. " "	3·68
20. " "	4·25
21. <i>Helianthus annuus</i>	3·94
22. <i>Helianthus tuberosus</i>	3·78
23. <i>Funkia sinensis</i>	5·91
24. <i>Allium Cepa</i>	3·76
25. <i>Heimerocallis fulva</i>	2·07
26. <i>Populus</i> (? sp.)	3·79
27. <i>Syringa vulgaris</i>	2·53
28. <i>Cotyledon Umbilicus</i>	4·61
29. <i>Humulus Lupulus</i> . Leaves	2·01
30. " " Strobiles with seeds (1) ..	9·60
31. " " " " (2) ..	7·95
32. " " " " (3) ..	6·00
33. " " Strobiles free from seeds ..	2·06
34. <i>Hymenophyllum demissum</i>	4·20
35. <i>Hydrocharis Morsus-ranæ</i>	0·267

We see from the above table that the foliage leaves of all the plants we have examined contain more or less diastase, but that the amount of this enzyme varies enormously in different plants and, within narrower limits, even in the same plant at different times

It will help us to realise the high diastatic activity of some of these leaves if we compare it with the hydrolytic power of *malt* acting under the same conditions.

In the case of *Pisum sativum*, we see that its foliage leaves contain a sufficient amount of diastase to convert 24 times its own dry weight of starch within the standard time of 48 hours. A comparison was made between this and a pale barley-malt of average diastatic capacity.

Maltose produced under standard conditions by

	Grams.	Ratio.
1. 10 grams of leaf	240·30	1·00
2. 10 grams of malt.	634·61	2·64

We are thus led to the remarkable conclusion that the diastatic activity of the leaf of *Pisum sativum* is between one half and one-third of the diastatic activity of an average barley malt. At the other end of the scale we have the leaves of *Hydrocharis Morsus-ranæ*, which can only effect the hydrolysis of 2·5 per cent. of their own dry weight under the standard conditions. We have in this case, however, to deal with a leaf containing a *very large amount of tannin*, and we have no doubt that on this account the number in our table is far too low as an expression of the diastatic activity of this plant.

It is a noteworthy fact that the *Leguminosæ* in the above table stand out pre-eminently for the high diastatic power of their leaves. The one exception is the *lupine*, and here again we found we had to deal with a leaf containing much tannin, a fact which, doubtless, explains the exception.

The high value found for the grass, No. 11, was no doubt due to the clover contained in the lawn-cuttings which were used.

In considering the very variable diastatic function of leaves, it is a matter of considerable interest to ascertain if there is any correlation between the average amount of *diastase* in the leaf and the facility with which any particular plant forms *starch* in its chloroplasts.

Unfortunately we do not possess at present a sufficient number of accurate observations to enable us to determine this point. The researches, however, of Sachs, A. Meyer, and others, have shown how enormously the leaves of various species of plants differ in their capacity for forming starch, and have given us certain data upon which something like a general opinion can be based.

A. Meyer (34) has generalised his observations by arranging the Natural Orders of the Dicotyledons which he has examined in five different classes, according to the amount of starch which they can store in their leaves under favourable conditions, this starch being

determined by the amount of iodine coloration which the strongly insolated leaves can exhibit. In the first class he puts the *Solanaceæ* and the *Papilionaceæ* as being able to form very large quantities of starch. Our own observations have not extended to any of the *Solanaceæ*, but it will be noticed in the table that all the *Papilionaceæ* examined are highly diastatic, pre-eminently so in fact. The one exception, the *lupine*, contains, as has been stated, a considerable amount of tannin which has, doubtless, obscured the result.

Amongst the Monocotyledons, it has been long known that certain of the *Liliaceæ* form little or no starch in their leaves. Some few of these, such as *Allium Cepa*, *Galanthus nivalis*, and *Hyacinthus orientalis*, appear to be unable, under any circumstances, to elaborate starch in their chloroplasts; whilst, on the other hand, certain other species, such as *Iris germanica*, which never contains starch under the most favourable natural conditions, produce it in their chloroplasts fairly readily when the leaves are bathed with an atmosphere containing 5 per cent. of carbon dioxide [*vide* Boehm (30)].

The Liliaceous plants are certainly very poor starch formers, and we find they are also very poor in diastase, especially those species which never produce starch.

Of the three Liliaceous plants given in the last table, Nos. 23, 24, 25, *Funkia sinensis* can produce a moderate amount of starch in its leaves, whilst *Allium Cepa* and *Hemerocallis fulva* are not starch producers. We notice that the first-named plant is considerably more diastatic than the two last.

Although these observations, as far as they go, strongly suggest that facility for starch production in a leaf is related to the occurrence of a diastase, which it is fair to assume takes part in the redissolution of that starch, they must be very much extended before we obtain proof of this proposition. We have in the leaf of the *Hydrocharis*, No. 35 of the table, a case which at first sight appears to point the other way. No plant is more active in its manufacture of leaf-starch, under favourable conditions, than is the *Hydrocharis Morsus-ranæ*, yet we see the diastase present in its leaf is smaller in amount than in any other case. The disturbing cause here, however, is doubtless, as has been already stated, the very large amount of tannin contained in some parts of the leaf, which inhibits the action of the diastase under the conditions of experiment, and gives an altogether erroneous idea of the amount of enzyme present.

Putting this case of the *Hydrocharis* aside, we have found in all the leaves we have examined, far more diastase than is requisite to transform within a moderate amount of time all the starch which the leaf can ever contain.

We believe that future work will confirm the existence of a relation

between starch-forming power of leaves and diastatic function, but, as we shall show later, we need not wait for such experiments in order to prove that the disappearance of starch in the leaf under natural conditions of growth is, in part at any rate, brought about by a soluble enzyme, and that it is not altogether directly conditioned by the vital processes of the protoplasm, as taught by Wortmann.

The excessively high diastatic power of the leaves of Leguminous plants is very remarkable, and is another instance of the exceptional properties which recent research has shown to belong to those plants.

We suspected that this property might in some way be correlated with the richness of the leaf in *nitrogen*, and consequently we have determined the percentage of nitrogen in the leaves of *Pisum sativum* and of *Tropæolum majus*, leaves whose diastatic capacity stand in the ratio of 240—5. The following results were obtained:—

	Percentage of nitrogen.
Leaves of <i>Pisum sativum</i>	3.58
„ <i>Tropæolum majus</i>	4.52

In point of fact the highly diastatic leaf contains less nitrogen than the other.

7. Periodic Variations of the Diastase of Leaves.

We have already referred to the fact that the diastatic function of leaves of the same species of plant varies within certain limits, as is shown in the case of *Tropæolum*, Nos. 12—20 of the table. We have now to enquire into the laws regulating these variations.

As our method of determining the relative amount of diastase in leaves is a very accurate one; if all precautions are taken, it becomes possible, by an examination of the leaves of the same plant plucked at different times of the day and under varying conditions of insolation, to determine if the leaf-diastase shows any periodic rise or fall in amount, and if this change takes place in response to any alteration of external conditions.

There are two ways of carrying out such experiments. Fair results can be obtained by plucking at stated times from the same plant a certain number of leaves of about the same size and age, and determining the diastase in these as usual, after drying at a low temperature; but a much greater degree of accuracy can be obtained by a “half-leaf method”; that is, by making the periodic examinations upon fractional parts of identically the same leaves. It is this

latter method which we have employed exclusively in the experiments we are about to describe.

As the life of the leaf is prolonged for some time after it is plucked, if special precautions are not taken, metabolic changes still continue in the leaf-cells during the slow process of drying at the low temperature of 30—35°, which it is necessary to employ in order to avoid destroying wholly or in part the activity of the diastase.

As already remarked, this continued metabolism results in a marked diminution in starch before the leaf-tissue is dry enough to arrest further change, and we have also found that a marked alteration is simultaneously brought about in the amount of leaf-diastase when slow drying is adopted.

It became necessary, therefore, to devise some plan by which the vitality of the leaf could be quickly arrested at the time of plucking, and which would fix the amount of diastase without changing it in any way.

Immersion of the leaf in alcohol first suggested itself, but this plan has only a limited application, because we find that, although leaves or parts of leaves, after they have been in alcohol for 24 hours, can be fairly well compared *inter se*, yet the actual amount of diastatic activity which they exhibit after such treatment is only a fractional part of that which the leaves possess if dried at once. In other words, the alcohol destroys a large portion of the diastase. This is illustrated by the following experiments.

A number of leaves of *Tropæolum majus* were plucked and divided into equal halves. One set of half-leaves was immersed in alcohol at once, the other being air-dried in the usual manner at 30—35°. After 24 hours a determination of the diastatic activity in the two sets of half leaves gave the following numbers:—

	Diastatic activity according to usual scale.
Half-leaves dried at once	9·64
„ previously immersed in alcohol.	3·18

Another experiment on other leaves gave 7·43 as the measure of the diastatic activity of the leaves dried at once, against 3·77 for those immersed in alcohol.

This influence of alcohol in weakening the activity of diastase is also apparent with dry as well as with fresh leaves, as shown below.

	Diastatic activity.
<i>Tropæolum</i> leaves dried at 30—35°	5·93
The same dried leaves after treatment with alcohol for (1) 24 hours	1·12
(2) 72 hours	1·20

The last experiment shows that the weakening action of alcohol on diastase soon attains a maximum. When this maximum has been attained, comparison of diastatic activity can be made, but we much prefer to kill the leaves as soon as plucked by immersing them for a few minutes in air charged with chloroform vapour. Leaves so treated can then be slowly dried at a low temperature, without any fear of altering the amount of either the starch or diastase which they contain at the time of plucking.

Comparative experiments on these lines very soon showed that in the case of plants growing under natural conditions, the amount of diastase in their leaves is subject to periodic fluctuation, and that these fluctuations are in some way or other dependent on the degree of illumination. Moreover, it was further observed that the conditions which are the most favourable for assimilation and for the formation of starch in the leaf are just those which are the least favourable for the accumulation of diastase. In other words, speaking in general terms, the tendency towards starch production in the leaf is inversely related to the accumulation of diastase.

The following three experiments, carried out by the "half-leaf method," illustrate this:—

Experiments with Tropæolum majus.

	Diastatic activity.	Increase of diastase per cent.
1. Sept. 10. Half-leaves plucked at 5 P.M.	1·963	—
" " at 10 P.M.	2·662	35·6
2. Sept. 22. Half-leaves plucked at 3 P.M.	1·100	—
" " at 11 P.M.	1·874	70·3
3. Sept. 28 } Half-leaves plucked at 4.15 P.M.	1·006	—
& 29 }		
" " at 5.30 A.M.	1·645	63·5

In the next experiment, *Tropæolum* leaves were plucked at 5 P.M. on a day when the conditions for assimilation had been favourable, starch being plentiful in the mesophyll. The lamina of each leaf was divided into the three equal parts *a*, *b*, *c*.

The portions (*a*) were placed in a chloroformed atmosphere for a few minutes, and then air-dried at 30—35°; the portions (*b*) were dried under exactly the same conditions, but without being previously chloroformed; whilst portions (*c*), which had been left attached to the leaf-stalk, were placed in the dark for 18 hours with their petioles immersed in water. The laminæ were then separated from the stalk, and afterwards chloroformed and dried exactly as in the other cases. On determining the diastase, the following numbers were obtained.

	Diastatic activity.	Increase in diastase per cent.
a. Portions of leaf chloroformed and dried.....	2.163	—
b. Portions dried without chloroform treatment	3.687	70.4
c. Portions depleted in dark for 18 hours, chloroformed, and dried.....	4.728	118.5

This experiment shows in a striking manner the rapid increase in diastase when the leaves are cut and placed in obscurity at a time when they are full of newly-assimilated products.

The application of the iodized-chloral test indicated a very considerable reduction in the amount of starch as the diastase increased.

(a) Did not contain less starch than those portions of the leaves which had been immersed in alcohol for purposes of comparison; (b) contained considerably less starch than (a), owing to a continued metabolism during the early period of drying.

In (c), which had been in the dark for 18 hours, the starch had completely disappeared, whilst the diastase had attained a maximum.

It is only necessary to record the results of one more experiment of this kind to show how the gradual accumulation of diastase takes place in the leaf, *pari passu* with the depletion of the leaf in starch and other products of assimilation. In this case we used the entire plants of *Hydrocharis Morsus-ranæ*, which were grown in shallow tubs of water under perfectly healthy conditions.

After the plants had been fully insolated, and the leaves were very full of starch, they were covered over, portions of the leaves being taken after they had been in darkness for 47 and 96 hours.

Results of Experiments with Hydrocharis Morsus-ranæ.

	Diastatic activity.	Increase in diastase per cent.
1. Leaves after full insolation	0.267	—
2. „ in darkness 47 hours	0.476	78.2
3. „ „ 96 „	0.676	153.1

In 2 the starch was about one-half of that in 1, whilst 3 contained, after 96 hours in the dark, but a mere trace of starch.

We have now to consider what explanation can be given of the accumulation of diastase in the leaf when the plant is placed in the dark. One possible explanation is the following:—The protoplasm of all the assimilating cells of the leaf-parenchyma, and of all the other

cells in which starch transitorily appears, may be supposed to be secreting a certain amount of diastase, this amount being constant under all conditions of insolation. As long as assimilation is going on with sufficient activity to maintain an excess of starch in the chloroplasts or amyloplasts, this diastase is being used up in re-dissolving the starch, and consequently does not tend to accumulate in the cell. Supposing, however, that the plant is placed in the dark, or in such a feeble light that assimilation cannot continue, then the starch diminishes in amount until it finally disappears, and there is in consequence a constantly diminishing draft upon the diastase, which now necessarily accumulates in the cell, not because it is produced faster by the living elements of the cell, but because less is used up. The increase of diastase in the dark is, on the above supposition, due merely to demand being less than supply.

One serious objection to this view is that the observed increase of diastase seems to be out of all proportion to the amount of starch dissolved. If this were the correct explanation, we should expect the actual increase of diastase to be much less than it is. Moreover, this view supposes a constant formation and re-solution of starch going on *simultaneously* in the same cell, and it is very difficult to imagine that solid starch-granules can be deposited from some such pre-existent state as soluble-starch at a time when the cell contains far more than sufficient diastase to rapidly hydrolyse that starch. We are aware that Sachs has imagined such a constant flux of starch existing in every actively assimilating cell, but hitherto no sufficient proof, or in fact proof of any kind, has been forthcoming that deposition and re-solution of starch can take place simultaneously within the same cell. Our own observations upon the comparatively large grained leaf-starch of *Hydrocharis Morsus-ranae*, a plant well suited for such observations, are altogether opposed to this view of Sachs. In examining the leaf-cells when assimilating vigorously, or after partial depletion in obscurity, wherever signs of dissolution could be detected in any single starch-grain it was always possible to detect signs of action on *all the other starch grains lying within the same cell*; in fact, no suggestion of simultaneous solution and deposition in the same cell could ever be obtained.

It is much to be desired that some experienced botanist would undertake a series of observations upon this point. It is only by keeping under continuous observation the starch-grains of a living cell that the problem can with certainty be solved. For experiments of this nature it has been suggested to us, by Dr. Marshall Ward, that certain of the Filmy Ferns might be appropriate. The fronds of some of these, such as *Hymenophyllum demissum*, are packed closely with chloroplasts containing relatively large spindle-shaped starch-grains,

and from the pellucid nature of the fronds and the fact that they consist of only one layer of cells, it certainly seems possible that the cell-contents could, by a little careful arrangement, be rendered capable of continuous observation.

Another more probable explanation of the observed increase of leaf-diestase in darkness was suggested by some observations which we have described in an earlier paper on the germination of the Gramineæ (44). We there showed that the scutellar epithelium is the diestase-secreting tissue *par excellence* of the embryo of the Grasses, and that this power of diestase secretion is inhibited in a very remarkable manner as long as the embryo is artificially supplied with solutions of certain nutritive carbohydrates, and is not obliged in consequence to obtain its nourishment from the reserve-materials of the endosperm. We have given our reasons for believing that the faculty of secreting diestase is so adapted to the requirements of the young plant as to be only exercised when actually required, that is, when the supply of soluble nutriment is deficient. The secretion of diestase was in fact regarded as more or less of a *starvation phenomenon*.

We believe that this is also the true explanation of the increased diastatic power of leaves when placed in the dark. As long as the conditions are favourable for assimilation, the leaf-cells are supplied with an abundance of newly assimilated materials, and so plentifully that the supply exceeds their powers of metabolism and translocation. The excess of nutritive material is in part at least deposited as starch. At this period there is little or no elaboration of diestase by the protoplasm, probably none at all in those cells in which starch deposition is in active progress. When the light fails, and assimilation falls off, the living cells speedily use up or translocate the excess of the soluble assimilative products, *e.g.*, cane-sugar, and begin to draw their supplies from the reserve of starch. To enable them to do this effectually, the somewhat starved protoplasm now commences to elaborate the needed diestase more rapidly, and this secretion becomes still more marked as the starvation point of the cell is neared.

The view we have expressed above is by no means unsupported by experimental evidence. We have already several times referred to the experiments of Böhm and of A. Meyer in which they were able to produce starch in the leaf chloroplasts in darkness, by supplying the leaves with various kinds of sugars. Exactly the same appearances are produced under these artificial conditions as when the leaf cells are naturally supplied with sugars which the chloroplasts have themselves formed by assimilation.

On fully considering these experiments, and frequently repeating them with success, it occurred to us that they might be made to yield

results which were confirmatory or otherwise of the "starvation hypothesis" we have enunciated above.

At 9 A.M., on August 14, eight healthy leaves of *Tropaeolum majus* were taken, and each leaf was divided into three equal parts, which were arranged in three equal series, *a*, *b*, and *c*.

- a*. Dried at once for diastase determination.
- b*. Cut into small fragments of about 1 sq. cm. area, and floated on water in the dark.
- c*. Cut into fragments as in 2, but floated on a solution of 5 per cent. dextrose, also in the dark.*

At the end of 24 hours *b* and *c* were washed and dried, and diastase determinations were made with all three samples.

	Diastatic activity (usual scale).
<i>a</i> . Parts of leaves dried at once	3.911
<i>b</i> . " " floated on water for 18 hours.	4.461
<i>c</i> . " " floated on dextrose solution for 18 hours	3.123

In the portion (*c*) which had been floated on dextrose solution, the mesophyll cells had received such an excess of nutriment as to deposit large quantities of starch in the chloroplasts, especially near the edges of the fragments, and this abundant supply of carbohydrate food had been accompanied by a marked *decrease* in the amount of diastase. On the other hand, the leaf fragments of *b*, which had been deprived altogether of nutriment, and had their mesophyll cells reduced to a state of starvation and autophagy by cultivation on water only, showed a very marked *increase* in the amount of contained diastase.

These facts all tend to show that the "starvation hypothesis" is capable of explaining the periodic fluctuation of diastase in the leaf, and we believe that when attention has been fully called to this explanation future research will extend its application to all cases of deposition and re-solution of starch within the vegetable cell, whether that starch be of a transitory or of a truly reserve nature.†

* This experiment was repeated in duplicate upon carefully-measured pieces of leaf in order to correct for differences of weight when the diastase determinations were made. The differences were, however, too small to require correction for increase or decrease in weight.

† We believe that the "starvation hypothesis" will also be found capable of explaining several obscure facts in animal as well as in vegetable physiology, notably the deposition and dissolution of *glycogen* in the liver. By some physiologists it is believed that the small amount of glycogen-hydrolysing enzyme discovered in

8. *Can Leaf-diastase act on Solid Starch?*

We have now fully considered Wortmann's first objection to the view that dissolution of leaf-starch in the living plant is conditioned by a soluble enzyme, an objection based upon the supposed fact that leaves contain little or no diastase, and we have shown that this part of his argument is certainly invalid.

We must now consider how much weight must be attached to Wortmann's other two objections, that, on the one hand, leaf-diastase, when it does occur, is of such a nature as to be incapable of acting on the solid starch-granule, and, on the other hand, that the conditions which arrest the respiratory functions of the cell always prevent the dissolution of the starch-granule.

In his endeavours to ascertain if leaves ever contain a diastatic enzyme capable of attacking and dissolving the solid starch-granule, Wortmann, as in his other experiments, used the filtered aqueous extracts of leaves. These, as we have already pointed out, are conditions under which it is difficult to demonstrate even the existence of diastase. If a proper method of experiment is used, it is not difficult to show that leaves contain a diastase fully capable, under favourable conditions, of attacking the solid starch-granules.

Freshly pounded leaves of *Helianthus tuberosus* were mixed with a little barley-starch, and the thin, soupy mass was digested at a temperature of 30—35°, with the addition of a little chloroform to prevent growth of microorganisms.

Within a day or two, signs of dissolution were very evident in the case of the injured and broken granules, which were rapidly disappearing, and the same indications also became apparent soon afterwards in the intact granules, which, however, during dissolution, did not present the phenomena of pitting and corrosion so evident when

the liver is altogether incommensurate with the discharge of glycogen from that organ. In fact, exactly the same arguments have been used against the view that glycogen owes its dissolution to a special enzyme as have been used by Wortmann and others against the similar view that leaf-starch does not owe its dissolution to diastase.

The attempts which have been made to demonstrate the existence of the glycogen-hydrolysing enzyme in the liver have only been partially successful, owing, in the first place to the imperfect methods of procedure for isolating the enzyme, and, secondly, to the fact that these attempts have been generally made when the hepatic cells contain their maximum amount of glycogen; in other words, when the cells are well supplied with nutriment. The period when the search for the glycogenic enzyme should be made is when the supply of carbohydrates in the portal blood is falling off in amount, and the hepatic cells are in consequence in process of consuming their reserve of glycogen. These are conditions of the partial cell-starvation which one might expect to be favourable for the maximum production of the enzyme in question, and for the successful demonstration of its presence.

barley-starch is acted upon by malt-diastrase. In this case, dissolution commenced *within the granule*, the most watery layers being first attacked, and was accompanied by no marked erosion of the outer layers. It was only after the action had been continued for several weeks that any normally "pitted" granules could be detected.

In another experiment, air-dried *Tropæolum* leaves were pounded and mixed with water, to which had been added a mixture of the following starches: (1) wheat-starch; (2) buckwheat-starch; (3) barley-starch. In this and all other similar experiments, a little chloroform was used as an antiseptic.

After digestion for three days, the wheat-starch and buckwheat-starch were much acted on, whilst the barley-starch resisted for 24 hours longer, and it was from 10 to 11 days before the last-mentioned starch was acted on to any great extent.

In another similar experiment, using the pounded air-dried leaves of *Pisum sativum* and wheat-starch at a temperature of 40°, a very decided action on the granules was visible within 48 hours.

In these and many other similar experiments, the action on the starch-granule by the leaf-diastrase was distinctly comparable in point of time with that produced under similar conditions by the diastrase of germinated grain, but the rapidity of this action still fell short of that observed in the mesophyll of the living leaf when this is placed in the dark.

When we attempt to imitate *in vitro* any of the chemical phenomena attending physiological processes, there is always a difficulty in obtaining conditions favourable for the carrying on of the reaction with anything like the readiness with which they occur in the living tissue. Nevertheless, in the particular case before us we have been able, to some extent, under well-selected artificial conditions, to approach the rapidity of dissolution of the solid starch-granule within the plant-cell.

Of all the commonly known starches, that of buckwheat, *Polygonum Fagopyrum*, resembles more closely in appearance and structure the transitory starch of leaves; and as, moreover, it has been shown, in the first place by Baranetzky, that this starch is most readily acted on by the diastrase of germinating grain, it seemed to us a favourable starch for determining the rapidity of action of leaf-diastrase.

Some carefully prepared buckwheat-starch was digested with water for a day or two at 30°, to ensure its taking up the maximum amount of water possible. Some air-dried leaves of *Pisum sativum*, which had been proved to be highly diastatic, were finely powdered and made up into a thin, soupy mass, (1) with water alone, and (2) with

water containing 0·006 per cent. of formic acid.* To each of these a little of the buckwheat starch was added, and the samples were digested at 30° under the usual antiseptic conditions.

Within *two hours* there were distinct signs of action visible on the starch-granules in both cases, and further examination at 6 and 10 hours from the commencement of the experiment showed a gradually increasing dissolution of the starch, which was decidedly more pronounced in the slightly acidified solution than in the other, to which no acid had been added.

The action on the starch showed itself in both cases by the formation in the first instance of a less refractive "vacuole" in the centre of the granule, and from this radiating fissures gradually extended to the exterior, under the influence of which the granules were rapidly broken up and dissolved. Many of the granules were completely disintegrated and dissolved within 10 hours of the commencement of the experiment.

The result of this experiment is as conclusive as it is striking. There can be no doubt that solid buckwheat-starch can be attacked by the diastase of some leaves with a rapidity approximating that under which the starch is observed to disappear in the living leaf-tissue, and that this action is favoured by a very slight acid reaction, such as vegetable cell-contents generally possess. These are facts strikingly inconsistent with Wortmann's sweeping assertion that leaves contain no diastase capable of attacking solid starch.

Attempts made in a similar manner to demonstrate the dissolution of the leaf-starch of any particular plant by the diastase of its own leaf were only partially successful.

The starch of the *Tropæolum* leaf is apparently acted on only with considerable difficulty by its own diastase under artificial conditions, and this is true also of the comparatively large-grained starch of *Hydrocharis Morsus-ranæ*.†

Both these starches, and especially that of *Hydrocharis*, are, like potato-starch, very resistant to the action of malt-diastase, yet, notwithstanding this, the *Hydrocharis* leaf-starch is decidedly acted upon by the leaf-diastase of *Pisum sativum*.

* It had been proved that acid of this great dilution was absolutely without any action on the starch-granule at the temperature of the experiment.

† At the time these experiments were made it had not been recognised that the tannin of the *Hydrocharis* had such a marked inhibitory action on the diastatic function. The experiments require repeating with this in view. We think it probable that this leaf-starch will be found much more readily acted on than we were led to believe.

9. *How far is the Disappearance of Leaf-starch due to the Living Protoplasm?*

It was doubtless his observations on the non-disappearance of the starch from a leaf when this was placed under conditions inimical to the respiration of the cell, which first led Wortmann to the belief that the dissolution of the starch is dependent solely on the action of the living protoplasm, and not in any way on the presence of a starch-dissolving enzyme.

Although fully prepared to admit the accuracy of most of the experiments made by Wortmann in this particular direction, the first one which he mentions as being favourable to his view is certainly open to much criticism. He states that he cut up a leaf of *Helianthus* containing abundance of starch, and that, after placing the moistened fragments in the dark for 17 hours, he found none of the starch had disappeared from the mesophyll. From this he concludes that the disappearance of starch under normal conditions is directly dependent upon the transport of starch from the leaf, and points out that this should not be the case if the solution of starch is brought about by an enzyme.

We have frequently repeated this experiment with cut leaves of *Tropæolum*, with quite different results. It is true that the disappearance of starch from a cut leaf, either when this is entire or divided into numerous pieces, is not so rapid as when the leaf remains attached to the plant, but solution of starch undoubtedly takes place under these conditions, and often to a very considerable extent.

Wortmann's experiments which show the non-disappearance of starch from the leaf when plants are placed in an atmosphere of carbon dioxide are very important, as are also those which show that the depletion of the leaf in starch goes on with much less rapidity if the respiration of the cells of the petiole is seriously interfered with by covering the epidermis with an impervious varnish.

We have not repeated the experiments in this form, since, whilst fully accepting their accuracy and admitting that they are suggestive of the conclusions drawn from them by Wortmann, we do not think they afford the absolutely conclusive evidence which their author attributes to them, and for the following reasons:—

When the leaf-cells are bathed with carbon dioxide, as in Wortmann's experiments, all their metabolic processes must entirely cease, and consequently the diastase within the cell necessarily becomes fixed in amount, this amount of enzyme being, as we have seen, at a minimum when the mesophyll is fully charged with starch.

As long as there is a deficiency of oxygen for the normal respira-

tion of the cell, both metabolism and transference of already metabolised products from cell to cell must be arrested; but since the protoplasm is not necessarily *killed* by this temporary deficiency of oxygen, the primordial utricle is not readily permeable, even to highly diffusible bodies, such as the crystallisable products of starch-transformation.

It seemed, therefore, under the conditions of Wortmann's experiments, that little or no disappearance of starch would be likely to take place, even if its solution was conditioned by a mere chemical function of a portion of the cell-contents, *i.e.*, the action of an enzyme; for it is manifest that, under these artificial conditions, the products of dissolution would speedily accumulate in the cell, and thus tend to retard further action.

It appeared to us that it would be better, in approaching this part of our subject experimentally, to treat the leaf-tissue by some method which, whilst exerting little or no inhibitory influence on any contained enzyme, would at the same time actually *kill* the protoplasm, and bring it into such a condition that the products of starch hydrolysis could readily pass outwards when the conditions for such diffusion were made favourable.

Our first experiments in this direction were made upon the leaves of *Tropaeolum majus*, plucked after full insolation, and *immersed in alcohol for a few hours*. When half-leaves, so treated, were well washed with water, and placed in a moistened state at 30–35°, or floated in water at a similar temperature, they could never be induced to show a diminution in their contained starch within any time at all commensurate with that in which complete dissolution takes place in the still living leaf after it has been plucked.

As we have already noted the fact that the diastatic function of leaves is materially lessened by contact with alcohol, this method was not considered satisfactory; consequently, in another series of experiments, the leaf was first killed by placing it for about two hours in an atmosphere well charged with *chloroform* vapour. Half of each leaf was then marked and placed at once in alcohol for comparison, whilst the other half-leaves were treated for varying periods under such conditions of moisture and temperature as were considered suitable for bringing about the dissolution of the starch by any diastase contained in the tissue. The experiments were varied by floating the leaves on, or immersing them in, water containing a little chloroform.

Although very numerous trials were made in this manner with leaves of *Tropaeolum*, *Hydrocharis*, and *Pisum sativum*, we never succeeded in satisfactorily demonstrating any distinct dissolution of starch in the killed leaf, unless temperatures were employed approaching the gelatinising point of the starch, a condition so far removed

from any occurring in nature, that we need but make a passing allusion to it.

Since diastase, as we know it outside the plant, is very indiffusible, it seems tolerably certain that any enzyme action in the dead leaf-cells, under the conditions we have just described, must be confined to the particular cells in which the enzyme occurs at the time the vital processes of the leaf are arrested. In the case of the living plant, the movements of the protoplasm, and its continuity from cell to cell, must facilitate the transference of enzymes, a transference which must cease after the life of the cell-aggregate has been destroyed.

Bearing this in mind, and also the important fact that cells fully charged with starch contain little or no diastase, it seemed necessary to repeat the above experiments on leaves which were in process of depletion of their starch, and in which it was, therefore, fair to assume that all or most of the cells contained diastase.

Leaves of *Tropaeolum* and of *Hydrocharis* were consequently partially depleted of their starch in darkness, after full insolation, the depletion being, in some cases, allowed to take place with the leaves still attached to the plant, in others after the leaves were plucked. Here again, after chloroforming the leaves and placing them under the most favourable conditions for inducing dissolution of the remaining starch, no indication of such an action could be obtained.

Apart entirely from any metabolic function of the cell, it is evident that the starch-grains embedded in the chloroplast of a living leaf-cell, whilst still surrounded and carried along by the streaming protoplasm, must be placed under conditions far more favourable to the action of any enzyme which the cell may contain than is the case when all motion of the protoplasm has been arrested by limitation of oxygen or any other means. In this latter case, if the enzyme is to reach the starch at all, it must be by a process of diffusion through the protoplasm, and more or less of the chloroplast itself.

It seemed, therefore, just possible that the negative results we have described might, after all, be attributable to the inability of the highly colloidal diastase to gain access to the starch, by the process of diffusion, with sufficient rapidity to make its presence apparent.

Hitherto we have not been in possession of any data enabling us to form an opinion upon the probability of this being the case. As it is here not a question of the rate of diffusion through a cell-membrane, but through the viscid protoplasm itself after death, we thought that an approximate solution of the problem might be obtained by determining the rate of diffusion of diastase in a gelatin mixture of sufficient strength to remain solid at ordinary temperatures.

To a 3 per cent. gelatin solution, which was on the point of solidifying, a small quantity of buckwheat-starch was added, the starch

being well distributed through the mixture, which was then allowed to solidify in small beakers. This particular starch was selected, owing to the rapidity with which it is visibly acted upon by diastase.

A cold, aqueous infusion of malt had been meanwhile prepared, by digesting 100 grams of finely-ground malt with 250 c.c. of water for 24 hours. A portion of the filtrate from this was mixed with its own volume of a 6 per cent. gelatin solution, and when the resulting mixture, containing 3 per cent. of gelatin, was on the point of solidification, it was poured on to the mixture of starch-gelatin. When completely set, there was a perfectly sharp line of division between the malt-extract gelatin above and the starch-gelatin below.

When the experiment had been continued sufficiently long, the small beaker containing the mixture was placed for a little time in ice-cold water to render it as hard as possible, and the jelly was turned out and cut into vertical prisms. The extent of the diffusion of diastase from the upper layer into the lower was determined by ascertaining microscopically the distance through which the starch-granules exhibited any visible action. This will, of course, be, strictly speaking, somewhat less than the distance penetrated by the diastase, but very little, as we are dealing with a starch which is acted on very quickly.

When the experiment was to be continued for more than 24 hours, both gelatin mixtures were rendered antiseptic with a little thymol or chloroform.

In an experiment conducted on these lines, we found that in 22 hours the diastase had penetrated into the lower layer exactly 3 mm., the diffusion having been at an average rate of *0.136 mm. per hour*. This rate of diffusion of the diastase was maintained with great uniformity for the five days during which the experiment lasted. At the end of 117 hours the diastase had penetrated to a total depth of 17 mm., which gives an average rate for the whole period of *0.145 mm. per hour*.

In another experiment, using a solution of *precipitated diastase* instead of malt-extract, the average rate of diffusion was *0.061 mm. per hour*, or about half that of the more active transforming agent of malt-extract.

The rate of diffusion of diastase through a semi-solid gelatin mixture is considerably more rapid than we expected to find it, and if, as seems probable, the diffusion of the enzyme through the dead protoplasm and contents of the cell-vacuole proceeds with anything like the same facility, it is evident that we cannot attribute the non-disappearance of starch from chloroformed leaves to the colloidal nature of the leaf-diastase. This is rendered still more evident if we consider the maximum distance through which any part of the enzyme would

have to travel for equal distribution through the palisade-cell of a *Tropæolum* leaf. The longer dimension of such a cell is about 0.1 mm., and the shorter 0.025 mm., so that, under the ordinary physical laws regulating the diffusion of diastase, a particle of this enzyme would be able to traverse the dead cell from end to end in a little more than half an hour.

Taking the whole of the evidence into consideration, we are compelled to admit that, at any rate, the first stages of the action on the starch-granules of a leaf are dependent upon the life of the protoplasm; but when we review all the facts, and give due weight to (1) the constant and abundant occurrence of diastase in leaves; (2) the apparent dependence of this diastase upon the occurrence of starch; (3) the remarkable periodicity of the increase and decrease of diastase; and (4) the correlation of this periodicity with the appearance and disappearance of starch, we certainly cannot accept Wortmann's view, that the dissolution of starch in the leaf is in no way conditioned by a starch-dissolving enzyme.

It is true that the starch-granules which occur in leaves, whilst capable of being attacked by some forms of leaf-diastase, show, as a rule, a great initial power of resistance to the hydrolysing influence of their own diastase. We know that some starches, *e.g.*, that of the potato, as long as their granules are intact, exhibit a similar resistance to the attack of even such active forms of diastase as those of germinating cereals. Yet the recent experiments of Prunet (61) leave but little doubt that during the growth of the potato-tuber diastase plays an important part in dissolving the reserve-starch. The initial resistance of many starches to dissolution seems to be due to some slightly different physical or chemical property in the outer layers of the granule, for when the granules are broken or injured in any way, even the most highly resistant starches give way to the solvent action of diastase (Brown and Heron, *Trans.*, 1879, 624).

This appears also to be the case with the leaf-starch of the plants we have examined; the first attack on the starch-granule has apparently to be made by the living elements of the cell, but although the vitality of the protoplasm may be essential to the *commencement* of the action, it seems almost certain that the hydrolysis of the starch-substance when it has been brought down to the stage of soluble-starch is continued and finished by the enzyme.*

If it is a fact that diastase plays an important part in breaking

* The comparative stability of the starch-granule when once formed within the cell, and the necessity for some direct influence of the protoplasm upon it before it can be brought under the chemical action of the enzymes of the cell, must be of distinct economic advantage to the plant, and has probably been a by no means unimportant factor in determining plant evolution.

down the starch of the living plant, it is manifest that we ought to find evidence of the existence of *maltose* in the leaf at certain periods.

The proof of this we have given in the second part of this paper. We look upon it as a fact of the highest importance, and one which, in conjunction with the other evidence we have given, establishes almost beyond doubt the view that the dissolution of starch in the leaf is mainly conditioned by a soluble enzyme, *diastase*.

PART II.—THE SUGARS OF THE LEAF.

10. *Historical.*

It has long been known that foliage leaves contain a small but variable amount of a substance capable of reducing Fehling's solution, and it has generally been assumed that the reducing substance is a *sugar*, although but very little evidence of this has ever been forthcoming.

Müller-Thurgau showed, in 1882 (26), that the amount of cupric-reducing substance, under certain conditions, increased in quantity in the leaf as the starch diminished, but the whole question of the nature of the cupric-reducing compounds of leaves has never been submitted to anything like an accurate investigation.

In 1883, R. Kayser (27), in a paper dealing with the occurrence in the plant organs of cane-sugar, and its products of transformation, attempted to separate the sugars present in the leaves of the vine, beet, and several other plants. The mixed sugars, which were found in the filtrate from the cell-sap after neutralising and precipitating with lead acetate, were examined by determining the cupric-reducing power of the liquid before and after inversion with an acid, the results being calculated for cane- and invert-sugar. An independent proof of the existence of cane-sugar in leaves was given by actually crystallising it out, and determining its opticity. Kayser considered that both his chemical and microchemical results indicate that the leaf-starch is converted into cane-sugar by a diastatic ferment, and that this cane-sugar is inverted during its wandering through the vessels of the leaf.

In discussing the manner in which the assimilated starch of the chlorophyll is carried into the petioles and stems of plants from the leaves, Sachs, in his 1884 paper (32), discusses the question of the chemical condition of the solution products. He has no new experimental facts to offer, but considers that the opinion which he has advocated for many years, and which is generally received, is the correct one, viz., that the wandering form of the starch is *sugar*.

This opinion is, as we have before stated, entirely based on the re-

duction of Fehling's solution, and assuming the reducing body to be a sugar, Sachs has given the results of observations indicating the variable quantity of sugar met with in the leaves under different conditions. He states that, in the case of vigorously growing leaves like those of potato, gourd, &c., but little or no sugar is found when the leaves are attached to the plant and are being actively depleted of their starch, but that the sugars accumulate if the leaves are plucked and placed with their petioles in water, since under these conditions the products of dissolution of the starch cannot escape from the leaf.

In his important paper of 1885, A. Meyer (34) seriously attempts to investigate the nature and amount of the sugars of the leaf, and to ascertain if the leaves of plants like *Allium Cepa*, which store no starch in their leaves, contain any other cupric-reducing carbohydrates at all comparable in amount with the starch of other leaves. The soluble carbohydrates were determined in the leaf-sap by ascertaining the reducing power before and after boiling with hydrochloric acid, and were expressed as "reducing" and "non-reducing" carbohydrates. The numbers which Meyer obtained indicate the interesting fact that the leaves of plants which store starch abundantly contain comparatively little of the reducing and non-reducing soluble carbohydrates, whilst on the other hand, leaves of plants like *Gentiana lutea*, *Iris germanica*, *Allium Cepa*, *Asphodelus luteus*, &c., which store little or no starch, accumulate relatively large stores of soluble reducing substances which are probably glucoses. The appearance and disappearance of these leaf carbohydrates when the plants are insolated or placed in darkness appear to be regulated by the same law as those which condition the appearance and disappearance of starch in other leaves.

In his attempt to determine the nature of the carbohydrates stored up by the leaves of *Allium Porrum*, Meyer gives the first proof of the existence of true sugars in the leaf. From the expressed sap of 20 kilos. of leaves he obtained, by means of a process based upon precipitation with ammonia and lead acetate, a sweet, transparent mass which could not be induced to crystallise. It possessed an optical activity of $[\alpha]_D -20^\circ$, approaching that of invert-sugar, but neither dextrose nor levulose could be obtained from it. In cupric-reducing power this substance agrees pretty well with dextrose. It yielded with phenylhydrazine a crystallisable compound melting at 204° .

From the leaves of *Yucca filamentosa*, Meyer obtained a body which he shows to be identical with the *sinistrin* of Schmiedeberg. From its described properties it is evidently *inulin*, which, in the leaf of the *Yucca*, takes the place of starch as a transitory reserve substance.

In 1885, Schimper (35), from his observations on various leaves and

the way in which they form starch when they are supplied artificially with solutions of sugars, concludes that the formation of glucose precedes that of starch in the process of assimilation, and that the starch is formed out of this glucose when the quantity of sugar in the cell exceeds a certain maximum, differing according to the kind of plant.

Keim, in 1891 (52), estimated the sugars in the leaves of the cherry. As this sugar was not found to reduce Fehling's solution until after inversion with acid, it was assumed to be cane-sugar.

11. *The Sugars of Tropæolum majus.*

As there seemed to be every probability that the sugars of foliage leaves vary considerably in their nature, there appeared to be a better chance of arriving at some conclusions as to the genetic relations of the various sugars to each other and to the starch if we confined our attention to the examination of the leaves of a single species of plant.

After some preliminary experiments with the leaves of potato and *Helianthus tuberosus*, it was finally decided that the leaves of *Tropæolum* afforded, on the whole, the best opportunity of investigation in this particular direction. Accordingly, during the summer and autumn of last year, we cultivated a considerable quantity of *Tropæolum majus*, and with the leaves of this plant most of our experiments were conducted.

Besides ascertaining the nature of the sugars in the leaf and their variations in amount, when the leaf was placed under special conditions, we hoped to discover the relation which each sugar bears to the primary assimilation products on the one hand, and to the leaf-starch on the other; to determine, in fact, which are the "up-grade" sugars towards starch, and to see if any indications were forthcoming of the existence in the leaf of "down-grade" sugars proceeding from the hydrolysis of starch, a fact of very great importance as bearing upon the physiological mechanism involved in the dissolution of starch in the living cell. It seemed probable also that the observations on the "down-grade" sugars might serve to support or not, as the case might be, the supposition of Sachs that the starch is in a continual and rapid state of flux, that, in fact, all the products of assimilation necessarily pass through the form of starch.

It was also expected that some light would be thrown upon the particular form in which the sugars wander from cell to cell in their passage out of the leaf into the stem, and upon the nature of the sugars which contribute the most readily to the processes of respiration and to new cell growth.

Although our experiments, as far as they have gone, have by no

means satisfactorily cleared up all these important points, we think we have, at any rate, indicated a probable solution of some of the problems, and that if the methods we have employed are carried out with the necessary care and patience by those who follow us, we shall obtain a much more complete knowledge of the complex metabolic processes of the leaf than we possess at the present time.

The only sugars which we have been able to discover in the leaves of *Tropæolum* are cane-sugar, dextrose, levulose, and maltose.* The total weight of the sugars, like that of the starch, is exceedingly variable in amount, and the proportion which the various sugars hold to each other is also subject to great fluctuations.

In the preliminary experiments made with the leaves of potato and of the Jerusalem artichoke, the expressed juice of the leaf was experimented upon, but as it was found that some of the sugars underwent a change during the preparation of the sap, and also that there was a difficulty in calculating the sugars back upon the dry leaf, this method was soon abandoned for the one which we now describe in detail.

The leaves are dried on wire-bottomed trays, ranged one above the other in an oven heated by steam. This process must be accomplished rapidly, and without allowing any opportunity for a continuation of the vital processes of the leaf.

The thoroughly dried leaves, reduced to a powder, are first completely extracted with ether, to remove fat and chlorophyll. A weighed quantity (generally 10 grams) is then extracted with about 50 c.c. of 80—85 per cent. alcohol for 24 hours at 40—45°. The alcoholic extract is poured off through a filter, and the residue extracted a second time with a similar quantity of alcohol. At the end of a further period of 24 hours, the liquid is again poured off, and the residue is thoroughly washed with warm alcohol by decantation. From the extract and washings the spirit is then distilled off, after the addition of a couple of drops of ammonia, to prevent any invertive action of the vegetable acids. The residue from the distillate, after the addition of water, is evaporated, to drive off all the alcohol, and is poured into a 100 c.c. flask; 1 c.c. of a strong solution of basic lead acetate is added, and the whole is made up to 100 c.c.

A small quantity of a dark, slimy matter separates on distilling and evaporating the alcoholic extract, and unless a little basic lead acetate is added, filtration is only effected with extreme difficulty. The lead

* Amongst other sugars for which the alcoholic extract was examined were the pentoses and substances yielding pentoses on hydrolysis; but it was found, on distilling a solution of the leaf-sugars with strong hydrochloric acid and testing the distillate for furfuraldehyde, that only the very slightest coloration was given with aniline acetate, thus showing the absence of any carbohydrates of the pentose group.

acetate also serves to remove the *tannin* taken up by the alcohol from the leaves, which would affect the subsequent determinations.

Experiment showed that the evaporated alcoholic extract gave a strong tannin reaction with iron salts, but that after treatment with basic lead acetate, no trace of tannin could be detected. The amount of tannin present in the leaves of *Tropæolum* is not very large, but in some leaves, as we have already stated, it is present in considerable quantity.

The precipitate produced by the lead acetate is filtered off, and the filtrate, after its volume is noted, is treated with SH_2 ; the precipitated lead sulphide is then filtered off and washed, the filtrate and washings are evaporated to remove SH_2 , and the residue is made up to the original volume.

The liquid thus obtained (usually 90 c.c.) contains the sugars from 10 grams of leaf in a state fit for determination; this is conducted as follows :—

1. The opticity and cupric-reducing power of the solution are determined in the usual manner.

2. A portion of the solution is inverted with a little prepared *invertase* at 50—55°, and the opticity and cupric-reducing power are again determined.

3. Another portion of the solution is digested with HCl in a water-bath, and the opticity and reducing power are again determined after three hours. In this part of the process the proportions recommended by Elion for the inversion of maltose were employed, viz., 50 c.c. of the 1 per cent. solution with 3 c.c. of concentrated HCl for three hours, the solution being then neutralised with sodium hydrate and made up to 100 c.c.

The increase in reducing power and decrease in opticity in (2), on treatment with *invertase*, give the amount of *cane-sugar* present.

The increase in reducing power and decrease in opticity in (3), on inversion with acid, give (after allowing for the cane-sugar already determined) the *maltose*.

We have here also sufficient data for calculating the amount of *dextrose* and *levulose*.

The method of calculation is somewhat complicated, and will be best understood from an actual example.

10 grams of dried *Tropæolum* leaves were treated as above, and the extract containing the sugars was made up to 100 c.c.

1. The original solution gave

- a. Optical activity in 100 mm. = +0·7 division.

- b. 100 c.c. reduced 1·397 grams CuO.

2. After inverting with *invertase* :—

- a. Optical activity in 100 mm. = -0.65 division.
- b. 100 c.c. reduced 2.544 grams CuO.

3. After inversion with *acid* and correcting for dilution :—

- a. Optical activity in 100 mm. = -0.80 division.
- b. 100 c.c. reduced 2.662 grams CuO.

The cane-sugar can be calculated in the first place from the difference in cupric-reducing power between (1) and (2).

$$2.544 - 1.397 = 1.147 \text{ grams CuO.}$$

Since 1 gram of cane-sugar yields 1.052 grams of invert-sugar, which will reduce 2.321 grams of CuO, the amount of cane-sugar in the 100 c.c. of solution will be expressed by the fraction $1.147/2.321 = 0.4942$ gram.

The inversion of this amount of cane-sugar would require a fall in opticity of 1.26 divisions of the scale of the polarimeter, and in the above experiment we found an actual fall of 1.35 divisions, so that the correspondence of the indications given by reduction and opticity is sufficiently close.

The maltose is calculated from the increase in cupric-reducing power between (2) and (3), thus:—

The actual increase in reducing power per 100 c.c. is $2.662 - 2.544 = 0.118$ gram CuO, and the maltose present in 100 c.c. of the original solution is $0.118/0.976 = 0.121$ gram, 0.976 representing the difference between the amount of CuO reduced by 1 gram of maltose and the CuO reduced by a quantity of dextrose, 1.052 gram, derived from 1 gram of maltose by hydrolysis.

The amount of maltose indicated by reduction, 0.121 gram per 100 c.c., requires a fall in opticity on hydrolysis of 0.28 division in the 100 mm. tube, 0.121×2.35 . This number 2.35 is the difference between the opticity due to 1 gram of maltose per 100 c.c., as against that due to 1.052 grams dextrose under similar conditions. The actual fall in opticity was 0.15 division.*

Having now determined the amounts of cane-sugar and maltose, it remains to ascertain the nature of the other sugars present in the original extract, and contributing their share to the total opticity and reducing power.

It is manifest that the total opticity and reducing power of the

* The fall of angle on inversion with acid was, for some unexplained reason, always somewhat less than it ought to be on the supposition that it was due only to the hydrolysis of maltose. This probably indicates the presence of a small quantity of a hydrolysable substance other than maltose, and with a less optical activity. The evidence of the existence of maltose is, as will be shown later on, not confined to these analyses.

sugars other than maltose and cane-sugar can be obtained by calculating, in the first place, the opticity due to the observed amounts of cane-sugar and maltose, on the one hand, and the cupric-reduction due to the observed maltose, on the other, and then deducting these values from the total opticity and reducing power of the original solution.

The constants used in the calculations are as follows.

1 gram maltose reduces 1.345 grams CuO.

1 „ „ per 100 c.c. gives in the 100 mm. tube a rotation of 3.905 scale-divisions.

1 gram of cane-sugar under similar conditions gives a rotation of 1.92 scale-divisions.

Original opticity of solution in 100 mm... 0.7 divisions.

Opticity due to 0.121 gram maltose = 0.472 } 1.42 „
 „ „ 0.494 „ cane-sugar = 0.948 }

Opticity of other sugars -0.72 „

Original cupric reduction of 100 c.c. of solution 1.397 gram CuO.

Cupric reduction due to 0.121 gram maltose 0.163 „ „

Cupric reduction due to other sugars..... 1.234 „ „

We have thus obtained an expression of the opticity and reducing power of the sugars other than cane-sugar and maltose, and since these sugars gave with phenylhydrazine acetate a glucosazone which did not differ from that given by dextrose or levulose, and, moreover, we were unable in these mixed sugars to obtain indications of any substance other than dextrose and levulose, we are justified in calculating the composition of these residual sugars in terms of these bodies.

The opticity in 100 mm. of -0.72 division, and the cupric reduction of 1.234 grams CuO per 100 c.c., point to a composition of *

* The formula used in this calculation is as follows:—

Let x = dextrose,
 y = levulose.

Then (1) $2.205x + 2.205y$ = grams of CuO reduced per 100 c.c.

(2) $1.48x - 2.76y$ = opticity in scale divisions in 100 mm. tube.

This simplified gives—

$$y = \text{CuO} \times 0.1583 - \text{opticity} \times 0.2358,$$

$$x = \frac{\text{CuO}}{2.205} - y.$$

In the above the opticity of 1 gram dextrose per 100 c.c. is, in the 100 mm. tube 1.48 scale divisions, whilst -2.76 is a similar value for levulose. The amount of

Dextrose	0.1946 gram per 100 c.c.
Levulose	0.3650 „ „

We have now reached the following final expression of the composition of the sugars in the original solution, expressed in grams per 100 c.c.

Cane-sugar	0.4942
Dextrose	0.1946
Levulose	0.3650
Maltose	0.1210
	<hr/>
	1.1748

Since the solution contains the sugars derived from 10 grams of dried leaves, the percentage of sugars in the leaf of this particular sample of *Tropæolum* is

Cane-sugar	4.94
Dextrose	1.95
Levulose	3.65
Maltose	1.21
	<hr/>
	11.75

In delicate experiments of this kind, of course very much depends upon the degree of accuracy with which polarimetric readings can be made. The instrument we employed was a "half-shade" polarimeter, with which the error of reading, when a clear colourless solution was taken, certainly did not exceed 0.05 of a scale-division.

The cupric reductions were all made in duplicate, and even then were often repeated, to ensure the greatest possible accuracy. The conditions under which the reducing powers were determined were similar to those we have described in our previous papers.

As an example of the concordant results which this method gives, when care is taken, we append the results of two separate analyses, A and B, of the same sample of the dried leaf of *Tropæolum majus*.

Percentages of Sugars in Tropæolum leaf.

	A.	B.
Cane-sugar	3.24	3.39
Dextrose	4.69	$\left. \begin{array}{l} 2.41 \\ 2.18 \end{array} \right\} 4.59$
Levulose		
Maltose	0.76	0.61
	<hr/>	<hr/>
	8.69	8.59

CuO reduced by 1 gram of levulose has, for the sake of simplicity, been assumed to be the same as that reduced by 1 gram of dextrose (2.205 grams), but we are not prepared to admit that these factors are strictly identical.

Although the analytical details which we have given of the determination of the leaf-sugars leave but little doubt that *maltose* is present in the leaf in variable quantities, it seemed desirable, nevertheless, in view of the immense importance which must necessarily be attached to the existence of this product of starch-hydrolysis, to obtain more direct evidence of its presence.

When the solution of the mixed leaf-sugars obtained from a large quantity of dry leaves of *Tropæolum* (200 to 300 grams), as described above, is treated with phenylhydrazine acetate, an abundant crop of crystals of glucosazone is obtained after digestion of the mixed liquids on the water-bath in the usual way. On filtering off the separated osazone, and allowing the filtrate to cool slowly, a crop of characteristic clusters of fine acicular crystals, resembling maltosazone in all respects, separates out. These two osazones were purified by recrystallisation from alcohol, in which solvent they exhibited the characteristic behaviour of glucosazone and maltosazone respectively, and the nitrogen was determined in each by Dumas' method. The following results were obtained :—

1st precipitate gave 15·71 per cent. nitrogen.

2nd ,, ,, 11·21 ,, ,,

and

Glucosazone requires 15·64 per cent. nitrogen.

Maltosazone ,, 10·77 ,, ,,

These numbers supply undoubted proof of the existence of *maltose* in the leaf-sugars, and its presence was still further confirmed by treating a solution of the mixed sugars, after complete inversion with invertase, with separated *glucose*. This is an enzyme discovered by Cuisinier in maize, and which has the power of hydrolysing maltose to dextrose (compare Morris, *Trans. Inst. of Brewing*, 1893, 6, 132).

So far as is known, *glucose* has no hydrolytic action upon any other carbohydrate than maltose, so that an increase in cupric-reducing power in a solution digested under favourable conditions with *glucose* may be taken as a proof of the existence of maltose in the solution.

When the mixed sugars of *Tropæolum*, after inversion with invertase, were digested with *glucose* at the optimum temperature, there was always a very large increase in cupric-reducing power, amounting generally to about 75 per cent. of the increase observed on digesting the same solution with dilute acid at 100° as previously described.

The importance of these cumulative proofs of the existence of maltose in leaves cannot be over-rated from a physiological point of view. It renders it almost certain that the dissolution of the starch of the leaf-chloroplasts is brought about by the hydrolysing enzyme

diastase, which we know is contained in the leaf-cells at the time the starch is disappearing, and that, at any rate, in the later stages of its dissolution, the disappearance of the starch need not be attributed to the obscure "vital" processes of the protoplasm.

As an indication of how variable is the amount of leaf-sugars in the same plant, we append another analysis of the leaves of *Tropæolum* plucked early in the day.

	Percentage of sugars on dry leaf.
Cane-sugar	1·655
Dextrose.....	0·319
Levulose.....	0·620
Maltose	0·813
<hr/>	
Total leaf-sugars	3·407

We now commenced a series of experiments designed with a special view to throw some light upon the genetic relations which the sugars bear to each other on the one hand, and to starch on the other. It was hoped that it might be possible to determine which sugar or sugars stand between the first products of assimilation and starch, and which of them must be regarded as derived from starch, either by direct chemical transformation or by intermediate metabolism. In other words, we sought to know which are the "up-grade" sugars towards starch, and which are the "down-grade" sugars derived from its solution and metabolism.

It was also hoped that some light would be thrown upon the position which cane-sugar occupies amongst the metabolites of the leaf, a position which is at present very obscure.

A considerable number of leaves of *Tropæolum majus* were picked at 5 A.M. on August 23rd, and were at once divided into two portions, *a* and *b*. Portion *a* was quickly dried in the steam chamber, whilst the cut leaves of *b* were placed with their petioles in water, and were exposed to sunshine for 12 hours. This was effected in water-tight wooden trays, with coarse wire gauze stretched over the top. Through the openings in the gauze the leaf-stalks were passed into the water below, the leaf-laminæ being arranged so as not to overlap each other.

After exposure to sunshine for 12 hours, the leaves of *b* were also quickly dried.

Another portion of leaves, *c*, which had been insolated also for 12 hours, but in this case, whilst still attached to the plant, were also picked at 5 P.M. and dried.

The starch and sugars in these three samples of leaves were determined in the manner described.

- a. Picked from plant at 5 A.M.
 b. Plucked leaves insolated on trays until 5 P.M.
 c. Leaves plucked from plant at 5 P.M.

Day fairly bright and sunny.

I. *Results of Analysis expressed in percentages on the Dry Leaf.*

	a.	b.	c.
Starch.....	1.23	3.91	4.59
Sugars—			
Cane-sugar.....	4.65	8.85	3.86
Dextrose.....	0.97	1.20	0.00
Levulose	2.99	6.44	0.39
Maltose	1.18	0.69	5.33
Total sugars per cent.	9.69	17.18	9.58

In another experiment the leaves of *Tropæolum* were plucked at 9 A.M. on a dull morning, and a further quantity was picked after insolation on the plant for seven hours, the day being bright and sunny.

a. Leaves picked at 9 A.M.

b. Leaves picked at 4 P.M.

The following numbers were obtained on analysis:—

II.

	a.	b.
Starch	3.24	4.22
Sugars—		
Cane-sugar	4.94	8.02
Dextrose	0.81	0.00
Levulose	4.78	1.57
Maltose	1.21	3.62
Total sugars per cent....	11.74	13.21

In Exp. I we see that in the cut leaves, b, which were insolated on the tray, the starch, although it has increased considerably in amount over a, has not reached that of the leaves c, which had been insolated for a similar period whilst still attached to the plant. This we have always found to be the case in our experiments; the production of starch in the cut leaf not going on with the same energy as it does when the leaf is still attached to the plant. This result is somewhat

contradictory to those recorded by Sachs, who, however, relied entirely upon the iodine-test for a rough estimate of the starch present.

But although the *starch* does not accumulate so fast in the cut leaf as it does when the leaf is on the plant, a further consideration of Exp. I shows that this is not the case as regards the *sugars*, but that these accumulate considerably in the leaf when their passage through the petiole into the stem is no longer possible.

We have already called attention to the great increase which takes place in the weight of a given area of the lamina of a leaf when the leaf is plucked and placed with its petiole in water, a fact which was first noticed by Sachs, and rightly attributed by him to the abnormal accumulation of assimilation products under these conditions. We now see that a considerable amount of this increase in weight is due to the temporary storage of sugars as well as of starch in the lamina, when the conditions prevent their free passage out of the leaf into the stem.

In turning once more to the results of Exp. I, we see that the total sugars of the leaf have nearly doubled in amount in the plucked and insulated leaves of *b*. This difference is mainly due to an increase in the amount of cane-sugar and of levulose, which have practically doubled in amount, whilst the dextrose has remained nearly constant, and the maltose has decreased to one half.

If the generally received opinion were correct, that a *glucose* is the principal "up-grade" sugar in the synthetic chain of assimilation, we should expect to find, with plucked and insulated leaves, an accumulation of a sugar of this class in the tissues of the leaf. Such, however, is not the case. It is true that we do find an accumulation of levulose, but we are by no means justified on this account in looking upon this sugar as an accumulated "up-grade" product, either towards starch or cane-sugar. It is just as probable that it may have been derived from the partial inversion of the cane-sugar, the equivalent quantity of dextrose, which was simultaneously produced, having been used up more readily than the levulose for the respiratory and other vital requirements of the cell. This latter supposition is rendered much more probable from the results of certain experiments which we must now describe, and which were designed with the specific object of determining the changes which take place in the starch and sugars of the living leaf-tissues when these substances are not being added to by the processes of assimilation.

A considerable number of *Tropæolum* leaves were plucked at the same time, and divided into two parts, one part being dried at once, the other being previously placed for a certain time in the dark, with the stems dipping into water. The starch and sugars were then determined in both.

III. *Leaves of Tropæolum, plucked in afternoon of a fairly sunny day.*

a. Leaves dried at once.

b. Leaves placed in the dark for 24 hours.

	a.	b.
Starch	3·693	2·980
Sugars—		
Cane-sugar	9·98	3·49
Dextrose	0·00	0·58
Levulose	1·41	3·46
Maltose	2·25	1·86
Total sugars per cent. ...	13·64	9·39

Total loss of sugars and starch in b = 4·96 per cent.

IV. In another experiment on the same lines, leaves of *Tropæolum* were taken after a bright warm day.

a. Leaves dried at once.

b. Leaves placed in the dark for 24 hours.

	a.	b.
Starch	5·425	0·906
Sugars—		
Cane-sugar	7·33	3·35
Dextrose	0·00	1·34
Levulose	2·11	3·76
Maltose	2·71	1·28
Total sugars per cent. ..	12·15	9·73

Total loss of sugars and starch in b = 6·93 per cent.

In both these cases we see that during the 24 hours the leaves have remained in the dark they have suffered a considerable loss both in sugars and in starch, amounting in Exp. III to 4·96 per cent. on the dry weight of the leaves, and in Exp. IV to as much as 6·93 per cent. There can be no doubt that this loss is due to the respiration of the leaf-cells. The amount of loss due to respiration has, as far as we know, never been determined for the leaves of *Tropæolum*, but we may obtain some idea of the value of this from Weber's experiments upon the leaves of *Helianthus*, in which he found the loss due to respiration equal to about 1·7 grams per square metre of leaf surface in 24 hours. Taking the average weight of a square metre of *Tropæolum* leaf at 25 grams, a number approximately correct according to

our own determinations, the loss of sugars in 24 hours from respiration is, in our two experiments, 1·24 grams and 1·7 grams per square metre.

Besides this actual loss of carbohydrates due to respiration, there has been a considerable alteration in the relative amounts of the sugars during the time the leaves have been in the dark. In the first place, the cane-sugar has diminished very considerably in both cases, in Exp. III the cane-sugar in the leaves which had been in the dark for 24 hours having fallen to about one-third of the amount present in the fresh leaves, and in Exp. IV to considerably less than one-half.

As this disappearance of cane-sugar has been accompanied by an increase in the amount of levulose in both cases, and there can be, of course, in this case no complication arising from assimilation, we must conclude that the cane-sugar has been inverted. That only a comparatively small part of the dextrose of this invert-sugar is found in the leaves is no doubt due to the fact that dextrose is more readily put under contribution for the respiratory processes of the cell than is levulose. Our experiments, in fact, enable us to form some idea of the relative facility with which the different kinds of sugars are used up by the cell in respiration.

If we assume, what is almost certainly a fact, that the starch which has disappeared from the leaf in the dark has been converted into maltose, and, what is still more certain, that the cane-sugar which has disappeared has been previously inverted, then we can calculate the composition of the mixed sugars which have been used up for cell requirements; they are as follows:—

Sugars used up in respiration, expressed as a percentage on dry leaf.	
In Exp. III—	
Dextrose	2·66
Levulose	1·19
Maltose	1·10
	<hr/>
	4·95
In Exp. IV—	
Dextrose	0·65
Levulose	0·34
Maltose	5·94
	<hr/>
	6·93

We must conclude from these experiments that maltose and dextrose are the sugars which contribute most to the respiratory requirements of the leaf-cell.

We must now revert once more to Exp. I, p. 669, where the sugars were allowed to accumulate in the leaf whilst the natural processes of assimilation were going on. In the light of the results obtained in Exps. III and IV, it seems almost certain that the levulose found in *b*, Exp. I is not a direct product of assimilation, but has been derived from the cane-sugar by inversion.

Looking at the results all round, they are, it seems to us, decidedly opposed to the view that either dextrose or levulose are the first sugars formed by assimilation, and point to the somewhat unexpected conclusion that, at any rate in the leaves of *Tropæolum*, cane-sugar is the first sugar to be synthesised by the assimilatory processes.

There seems every reason to believe that this cane-sugar, which may be regarded as the starting point of all the metabolic changes taking place in the leaf, functions in the first place as a temporary reserve-material, and accumulates in the cell-sap of the leaf-parenchyma when the processes of assimilation are proceeding vigorously.

When the degree of concentration of the cane-sugar in the cell-sap and protoplasm exceeds a certain amount, which probably varies with the species of plant, starch commences to be elaborated by the chloroplasts, this starch forming a somewhat more stable and permanent reserve-material than the cane-sugar, a reserve to be drawn upon when the more easily metabolised cane-sugar has been partially used up.

That the starch which is formed by the chloroplasts is, strictly speaking, not autochthonous, but owes its origin to antecedent cane-sugar, is rendered probable not only from a consideration of the results we have described, but also from experiments upon the artificial nutrition of leaves by solutions of carbohydrates, and from certain results which we described in 1890 on the starch-producing powers of some of the sugars when used as a nutrient for the cultivation of embryos of barley (Trans., 1890, 484). In both these cases, cane-sugar was found to far surpass all other carbohydrates in bringing about the production of starch in the tissue of the leaf or young plant; so much so, in fact, as to suggest that under natural conditions of plant growth cane-sugar is an antecedent of the formation of starch by the chloroplasts, a conclusion which we think is favoured by our more recent work.

As regards the particular form in which the carbohydrates wander from cell to cell in the leaf, and finally pass through the leaf stalk into the stem, we think our analyses point to the cane-sugar being translocated as dextrose and levulose, and the starch as maltose, the latter process only taking place when the starvation of the cell has induced the dissolution of the starch in the manner already described.

In the case of the invert-sugar, the dextrose is, as we have seen,

more quickly used up for respiration, and possibly also for tissue forming, than is the levulose; therefore it is highly probable that, under natural conditions, a larger amount of levulose than of dextrose passes out of the leaf into the stem in a given time.

Knowing as we do how enormous is the resistance which living protoplasm presents to the ordinary physical processes of diffusion, it seems highly improbable that the wandering of the sugars in the tissues of plants is dependent altogether upon osmosis. It is, doubtless, to the continuity of the protoplasm from cell to cell, which may now be regarded as a well established fact, that we must look for a full explanation of those rapid translocations of certain substances which we know take place in the living plant. That diffusibility is, however, a factor of importance cannot, we think, be doubted, when we regard the nature of those substances which up to the present time have been recognised as wandering metabolites.

ADDENDUM.

Since writing the above we have been able to determine the diastatic activity of the leaves of three plants belonging to the *Solanaceæ*. The results are given in the same terms as those employed in the table on p. 641.

	Diastatic activity.
<i>Solanum tuberosum</i>	8.163
<i>Nicotiana affinis</i>	7.524
<i>Lycopersicum esculentum</i>	6.569

Although the leaves of these *Solanaceæ* are much less diastatically active than those of the *Leguminosæ* we have examined, they immediately follow this Natural Order in the amount of starch-dissolving enzyme they contain.

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